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(54) Title: RESTENOSIS/ATHEROSCLEROSIS DIAGNOSIS, PROPHYLAXIS AND THERAPY

(57) Abstract

Disclosed and claimed are compositions and methods for therapy and/or prevention of restenosis and/or atherosclerosis. The compositions can include an agent for decreasing viral load of cytomegalovirus, such as an immunological composition or vaccine against cytomegalovirus (CMV) containing at least one epitope of interest of CMV and/or an expression system which expresses at least one epitope of interest of CMV. Such compositions can include at least one epitope of p53. Alternatively, the compositions can include at least one epitope of p53 and/or an expression system which expresses the epitope. The methods can include administering the compositions to a patient in need of such therapy and/or prevention. Additionally, compositions and methods for diagnosing atherosclerosis and/or restenosis, or susceptibility thereto, including screening a sample from a patient for antibodies to CMV and/or CMV proteins and/or screening a sample from a patient for specific viral proteins that predict whether the virus has been reactivated and/or antibodies thereto and/or detecting whether CMV nucleic acid, e.g., mRNA is present in peripheral blood monocytes (PBMCs) and/or detecting a cellular–mediated immune response to CMV peptides or proteins is present and/or HLA phenotyping and/or HLA genotyping. Embodiments can include a skin test.

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TITLE OF THE INVENTION

RESTENOSIS/ATHEROSCLEROSIS DIAGNOSIS, PROPHYLAXIS AND THERAPY

FIELD OF THE INVENTION

This application claims priority from U.S. application Serial No. 08/796,101, filed February 5, 1997, incorporated herein by reference.

The present invention relates to compositions and methods for the diagnosis, prophylaxis and/or therapy of restenosis and/or atherosclerosis.

The present invention relates to the use of an agent for decreasing viral load, e.g., an immunological composition, preferably a vaccine, against cytomegalovirus and/or p53 for therapy for restenosis and/or atherosclerosis; and, to a method for providing therapy for restenosis and/or atherosclerosis comprising administering the agent for decreasing viral load, e.g., an immunological composition or vaccine, against cytomegalovirus (CMV) and/or p53.

"Viral load" and "virus load", as used herein, can have their art-recognized definitions, and can refer to active virus, e.g. virus in circulation or infectious, non-dormant virus, as well as virus which is latent or dormant awaiting reactivation or reactivating, or virus which is having an abortive replication cycle. While restenosing patients may not have any increase in IgG or IgM, Applicants, without wishing to necessarily be bound by any one particular theory, submit that viral reactivation following angioplasty/atherectomy can occur; and, that this viral reactivation, in some instances, may only proceed as far as the turn-on of IE genes and not up to viremia. Thus, "viral activation" is included in "viral load" and "virus load" herein. Further, "atherectomy" is included in "angioplasty" herein.

The CMV antigen can derive from any CMV protein, including immediate early (IE), early, or late gene products. The antigen can be the entire protein or an antigenic portion thereof.

The p53 can be wild-type or a mutant, e.g., full-length p53 or a truncated antigenic portion thereof.

The antigen(s) can be derived recombinantly, e.g., from expression by a virus, bacteria, or plasmid, *in vitro*, with subsequent isolation and purification; or from expression by a recombinant *in vivo*. Preferred expression systems include generally adenovirus, baculovirus, poxvirus, and DNA vector systems. For *in vivo* use, a recombinant adenovirus or poxvirus, such as a vaccinia virus or avipox virus (e.g., canarypox virus), or a DNA vector system are preferred; but, any suitable vector system, including naked DNA, may be employed. Indeed, as herpesvirus vectors are known, a replication-deficient herpesvirus vector, e.g., a replication-defective HSV or CMV vector could even be used in embodiments of the invention.

The invention thus relates to stimulating an immune response, preferably a cellular immune response, directed against CMV and/or p53 to inhibit or prevent restenosis and/or atherosclerosis and/or smooth muscle proliferation. Such a response can cause cell lysis and thus inhibition of smooth muscle cell proliferation and/or inhibition of atherosclerosis and/or restenosis. Thus, the invention relates to methods for inducing cell lysis of smooth muscle cells and/or inhibition of smooth muscle cell proliferation to treat or prevent restenosis and/or atherosclerosis.

The administration of the immunological composition or vaccine can be before or at the time of angioplasty, e.g., coronary and/or peripheral angioplasty, to prevent the development of restenosis, or independently of angioplasty, to provide treatment for atherosclerosis. It can also be administered any time during the lifetime of the individual, from childhood to adulthood, to prevent the development or progression of atherosclerosis. Thus, the invention relates to a therapeutic method for treatment of atherosclerosis and/or restenosis.

The immunological composition or vaccine can be administered alone or with additional therapeutic treatment; and, the invention further relates to additional methods for therapeutic treatment of restenosis and/or atherosclerosis.

The additional therapeutic treatment can comprise therapy for decreasing viral burden, e.g., the administration of: antioxidants which inhibit the replication of CMV and the cytopathic effect of viral infection, and/or compositions which reduce the transcriptional activity of CMV (transcriptional activity reducer) and/or compositions which decrease reactive oxygen species (ROS) generated by the arachidonic cascade and/or the xanthine/xanthine oxidase system (ROS reducer). Additionally or alternatively, the additional therapeutic treatment can comprise administration of an antiviral agent such as gancyclovir and/or acyclovir.

Thus, the invention still further relates to a method for treatment of atherosclerosis and/or restenosis comprising administering a sufficient dose or doses of at least one agent for decreasing viral burden and/or directed to interfering with SMC proliferation, e.g., antioxidant which inhibits the cytopathic effect of viral infection and/or transcriptional activity reducer and/or ROS reducer, either alone, or in conjunction with the aforementioned immunological composition or vaccine therapy.

The antioxidant can be one or more of Vitamin C, Vitamin E, NAC, PDTC, and the like.

The transcriptional activity reducer can be an antiviral drug such as gancyclovir and/or acyclovir (which interfere with viral replication), and/or an antioxidant, or the like.

The ROS reducer can be aspirin (acetylsalicylic acid) or a derivative thereof, ASA, Indomethacin, oxypurinol, and the like.

Accordingly, the invention additionally relates to a method for treating restenosis and/or atherosclerosis

comprising, after angioplasty: administering a sufficient dose or doses of an immunological composition, preferably a vaccine, against CMV and/or p53; or administering a sufficient dose or doses of an immunological composition, preferably a vaccine, against CMV and/or p53, with or without a sufficient dose or doses of an antioxidant which inhibits viral infection and/or the cytopathic effect of viral infection and/or transcriptional activity reducer and/or ROS reducer; or administering a sufficient dose or doses of one or more antioxidant which inhibits viral replication and/or the cytopathic effect of viral infection and/or transcriptional activity reducer and/or ROS reducer.

The compositions administered after angioplasty can be used before angioplasty, to prevent, i.e., as a prophylaxis against, restenosis and/or atherosclerosis.

Accordingly, the invention relates to a method for preventing restenosis and/or atherosclerosis comprising, before angioplasty: administering a sufficient dose or doses of an immunological composition, preferably a vaccine, against CMV and/or p53; or administering a sufficient dose or doses of an immunological composition, preferably a vaccine, against CMV and/or p53 with or without a sufficient dose or doses of at least one composition for decreasing viral burden and/or directed to interfering with SMC proliferation, e.g., antioxidant which inhibits the cytopathic effect of viral infection and/or transcriptional activity reducer and/or ROS reducer; or administering a sufficient dose or doses of at least one agent for decreasing viral burden and/or directed to interfering with SMC proliferation, e.g., antioxidant which inhibits the cytopathic effect of viral infection and/or transcriptional activity reducer and/or ROS reducer. Thus, the invention can relate to treatment or prophylaxis directed at both decreasing viral loads, and decreasing SMC proliferation.

Interesting therapeutic or prophylactic compositions and methods of the invention relate to

recombinants, especially for *in vivo* use, expressing a CMV antigen or portion thereof, or p53 or a portion thereof, or a combination of a CMV antigen or portion thereof and p53 or a portion thereof. These recombinants can additionally express or be used in conjunction with another form of molecular based therapy, e.g., expression of cytotoxic molecules to inhibit proliferation of smooth muscle cells, gene therapy, or antisense strategies to inhibit expression of gene products for cell proliferation. Thus, an embodiment can be providing treatment directed at decreasing viral load and treatment directed at reducing SMC proliferation.

Accordingly in certain aspects, the present invention relates to vaccine or immunological compositions for treatment or prophylaxis of restenosis and/or atherosclerosis, including compositions containing a CMV antigen or portion thereof, e.g., IE1, IE2, IE1 and IE2, or antigenic portions thereof or any other CMV antigens from IE, early, or late gene products, p53 or an antigenic portion thereof, or a CMV antigen or portion thereof and p53 or portion thereof. The present invention can include compositions containing naked DNA expressing the CMV antigen or protion thereof, or a recombinant or recombinants expressing the CMV antigen or portion thereof and/or p53 or an antigenic portion thereof or such an antigen or portion thereof from recombinant expression. The present invention further includes uses of such compositions with additional treatment or therapy, including compositions containing a recombinant or recombinants expressing a component of such additional treatment or therapy or co-expressing the component of such additional treatment or therapy with the CMV antigen or portion thereof and/or p53 or an antigenic portion thereof, and methods of making and using such compositions. Naked DNA or recombinants used in the present invention can be of varied type; for instance, one antigen or portion thereof or component of additional therapy may be expressed in one

type of system, and another antigen or portion thereof or component of additional therapy (if present) may be from the same, or a different, system.

The method for diagnosis to ascertain a susceptibility to atherosclerosis and/or restenosis can comprise immunologically detecting CMV antibodies, either against the whole or any part of the virus, or preferably against specific viral proteins that more specifically reflect reactivation of the virus such as IE72, IE84, IE55 and the like. The immunologically detecting can be by ELISA and/or immunoblotting. Alternatively, detection can be for the CMV antigen.

The method can include, in addition or alternatively to detecting the neutralizing antibodies or antigens elicited thereby, detecting whether CMV mRNA is present in peripheral blood monocytes (PBMCs), e.g., by PCR (such as RT-PCR) and/or detecting whether a cellular-mediated immune response to CMV peptides or proteins is present, e.g., whether PBMCs recognize and/or respond to CMV peptides or proteins.

This aspect of the invention can relate to a skin test whereby the CMV proteins or peptides are administered subcutaneously or intradermally or intramuscularly, which reflects the patient's capacity to mount a cellular-mediated response targeted to the CMV proteins or peptides. A negative vs. a positive skin test for patients with prior CMV infection reflects the capacity to not develop, or to develop, respectively, a cell-mediated immune response to CMV. Such a test allows a prediction of who is susceptible and who is resistant to atherosclerosis and/or restenosis.

This aspect of the invention can relate more generally to presenting the patient's PBMCs with CMV proteins or peptides and measuring either the proliferative response of the cells or the cytokine profile to determine whether there is a dominant Th1 (e.g., IL-2, IFN-12, IFNγ) or Th2 (IL-4, IL-10) response.

This aspect of the invention can also relate to

HLA phenotyping and/or HLA genotyping, as such phenotyping and/or genotyping can be used to predict the susceptibility to CMV-induced vascular disease.

This aspect of the invention can further relate to detection of p53. CMV interacts with p53 in smooth muscle cells (SMCs). p53 present in increased amounts binds to MHC Class I antigens in the SMCs and is processed and presented at the cell surface at an increased rate, resulting in stimulation of T cell response, underlying the antibody responses (whereas normal p53 is immunologically silent). Increased or steady state levels of p53 are present in cancers or when viral oncoproteins bind to p53 (as is the case with CMV).

Thus, the diagnostic method can comprise screening a sample from a patient (e.g., sera, blood, SMCs, etc.) for antibodies to CMV. The method can further comprise: screening a sample from a patient for specific viral proteins or antibodies thereto that are more specific predictors of whether the virus has been reactivated such as IE72, IE84, IE55 and the like; and/or detecting whether CMV mRNA is present in PBMCs, e.g., by PCR (such as RT-PCR); and/or detecting whether a cellular-mediated immune response to CMV peptides or proteins is present, e.g., whether PBMCs recognize and/or respond to CMV peptides or proteins, e.g., by administering a CMV skin test by administering CMV proteins or peptides intradermally or subcutaneously or intramuscularly and ascertaining the result of the skin test and/or presenting CMV proteins or peptides to a patient's PBMCs and measuring either the proliferative response of the cells (PMBCs) or the cytokine profile; and/or HLA phenotyping and/or HLA genotyping; and optionally screening a sample from a patient (e.g., sera, blood, SMCs, lesions,) for p53.

The initial screening for antibodies to CMV may optionally be omitted, such that the diagnostic method can comprise: screening a sample from a patient for specific viral proteins that predict whether the virus has been

reactivated such as IE72, IE84, IE55 and the like; and/or detecting whether CMV mRNA is present in PBMCs, e.g., by PCR (such as RT-PCR); and/or detecting whether a cellular-mediated immune response to CMV peptides or proteins is present, e.g., whether PBMCs recognize and/or respond to CMV peptides or proteins, e.g., by administering a CMV skin test by administering CMV proteins or peptides intradermally or subcutaneously or intramuscularly and ascertaining the result of the skin test and/or presenting CMV proteins or peptides to a patient's PBMCs and measuring either the proliferative response of the cells (PMBCs) or the cytokine profile; and/or HLA phenotyping and/or HLA genotyping; and optionally screening a sample from a patient (e.g., sera, blood, SMCs, lesions, etc.) for p53.

The diagnostic method of the invention can also be used to test for stratification of atherosclerosis and/or restenosis risk factors.

The CMV proteins or peptides can be purified CMV proteins or peptides from lysates of cells previously infected with CMV, or from recombinant expression of the CMV proteins or peptides. Antibodies to such may also be used in diagnostic and therapeutic and/or preventative composition and methods of the invention. And, the CMV in the various aspects to which the invention pertains can be of any suitable cytomegalovirus, including, human CMV (HCMV) murine CMV (MCMV) or rat CMV (RCMV) origin, with HCMV and RCMV embodiments preferred.

Various documents are cited in the following text, or in a reference section preceding the claims. Each of the documents cited herein, and each of the references cited in each of those various documents, is hereby incorporated herein by reference. None of the documents cited in the following text is admitted to be prior art with respect to the present invention.

BACKGROUND OF THE INVENTION

As discussed generally by Jean Marx at page 320 of Science, Vol. 265 (July 15, 1994), each year about

330,000 patients in the United States undergo coronary and/or peripheral angioplasty, a procedure designed to open up blood vessels, e.g., coronary arteries, clogged by dangerous atherosclerotic plaques (atherosclerosis) and thereby restore normal blood flow. For a majority of these patients, the operation works as intended. Nearly 33% of these patients (and maybe more by some accounts), however, develop restenosis, wherein the treated arteries become quickly clogged again. These patients are no better off, and sometimes worse off, than they were before angioplasty. Excessive proliferation of smooth muscle cells in blood vessel walls contributes to restenosis.

Improvements in the therapy, prophylaxis and diagnosis of restenosis and/or atherosclerosis, especially in compositions therefore and methods thereof, would be an advance over the state of the art.

In 1950, Patterson and Cottral, in Arch. Pathol. 1950; 49:699, called attention to the development of coronary atherosclerosis in chickens ill with Marek's lymphomatosis, the etiological agent of which was subsequently discovered to be a herpesvirus now known as Marek's Disease Virus.

Melnick et al. in European Heart Journal (1993) 14 (Supplement K), 30-38, and BioEssays Vol. 17, No. 10 pp. 899-903 (1995) report that the finding in chickens prompted studies of human herpesviruses with respect to human atherosclerosis.

In Melnick et al., European Heart Journal, *supra*, circumstantial evidence for involvement of CMV is presented. This evidence includes finding CMV antigen and nucleic acid sequences in arterial smooth muscle cells of humans, seroepidemiological studies showing high levels of CMV antibodies found associated with clinically manifest atherosclerotic disease, suggesting that a periodically activated latent infection or a continuously active infection is present in patients with atherosclerosis. However, the viral genome, but not the infectious virus,

was found in arterial cells, leading the authors to assert that the artery itself may be the site of CMV latency. The authors caution that their observations do not demonstrate that viruses have a role in the pathogenesis of atherosclerosis.

In Melnick et al., BioEssays, *supra*, the authors report that antigens and nucleic acid sequences of CMV, a widespread member of the herpesvirus family, were found in arterial lesions in human atherosclerosis; but, infectious virus has not been observed. In atherosclerosis patients, high levels of CMV antibodies are present, suggesting the presence of virus that had been activated from a latent state.

There is no teaching or suggestion in Melnick et al., BioEssays, *supra*, of any particular CMV vaccine or any particular strategy for treatment, prevention or diagnosis of restenosis or atherosclerosis.

Speir et al., Science 265:391-394 (July 15, 1994) postulate that restenosis may be triggered by activation of latent CMV, e.g., by angioplasty-induced injury to the vessel wall, that causes multiple cellular changes and predispose SMCs to proliferate. For instance, Speir et al. postulate that CMV protein IE84 combines with and inactivates p53 in smooth muscle cells, which, in turn could predispose the cells towards increased growth, analogous to the way p53 inactivation is believed to contribute to the formation of malignant tumors. This CMV-mediated inhibition of p53, assert Speir et al., may in part explain the monoclonality observed in some atherosclerotic lesions (see Benditt and Benditt, PNAS USA 70: 1753 (1973)).

As Jean Marx, *supra*, observed, the Speir et al. hypothesis is just one of many potential mechanisms by which the virus may produce restenotic lesions. Jean Marx, *supra*, further observed that CMV activation cannot explain all cases of restenosis, as signs of a CMV-p53 interaction have not been found in about 67% of the restenosis samples.

Golubev et al., U.S. Patent No. 5,534,258 (not admitted to be prior art), relates to four polypeptides from certain herpesviruses; specifically two polypeptides from HSV-1, and two polypeptides from CMV. Golubev et al., without any data, speculates that this shotgun approach of a combination of all four of these polypeptides, in equal proportion, is a prophylactic vaccine against pathogenic development of atherosclerotic plaque. No protection data is presented.

Literature involving CMV and/or restenosis and/or atherosclerosis, as discussed above likewise fails to teach or suggest any therapy or prophylaxis or any detection methods, or any compositions therefor, for restenosis and/or atherosclerosis, as in the present invention. Indeed, heretofore there had not been a definitive teaching or suggestion in the art of a relation between the presence of antibodies to CMV at the time of angioplasty, indicating prior exposure to CMV, and the subsequent development of restenosis. And, even if, *assuming arguendo* (with no admission), one asserted some sort of teaching or suggestion of any relation between CMV or antibodies thereto and restenosis and/or atherosclerosis, there is still a failure to teach or suggest any therapy or prophylaxis or any detection methods, or any compositions therefor, for restenosis and/or atherosclerosis, as in the present invention.

It would indeed be an advance in the art to show a connection between CMV and restenosis and/or atherosclerosis, especially mechanisms involving the virus, including such as the virus, by inhibiting either the capacity of p53 to block cell cycle progression, or its capacity to initiate apoptosis, enhances SMC accumulation and thereby facilitates development of restenotic lesions, as herein.

Indeed, it is believed that heretofore there has been no evidence linking viremia and angioplasty, such as balloon angioplasty, and subsequent restenosis in humans,

e.g., no boost of immune response, such that there is *a fortiori* no teaching or suggestion of any prophylaxis or treatment for restenosis and/or atherosclerosis or compositions therefor or methods for making such compositions.

OBJECTS AND SUMMARY OF THE INVENTION

It is therefore an object of the invention to provide methods and compositions for the diagnosis of, prophylaxis of and/or therapy for restenosis and/or atherosclerosis.

It is yet a further object of the invention to provide such methods and compositions for prophylaxis and/or therapy which comprise an agent for decreasing viral load, e.g., a vaccine or immunological compositions.

It is a still further object of the invention to provide such methods and compositions including gene products from *in vitro* and/or *in vivo* expression from plasmid DNA, or a vector system, such as a recombinant viral and/or DNA expression system.

It is yet another object to provide such methods and compositions wherein the gene products comprise a CMV antigen, e.g., IE1 and/or IE2 or a portion thereof; gB; gB with transmembrane deleted therefrom; gH; gL; pp150; pp65; IE1 with amino acids 2-32 deleted therefrom; IE1 with amino acids 292-319 deleted therefrom; IE1 exon 4 segment; gB and gH; gB and pp65; gB, gH and pp65; gB, gH, pp65 and IE1 exon 4 segment; gB, gH, pp65, pp150, and IE1 exon 4 segment; gB, gH, pp65 and pp150; gB, gH, gL, pp65, pp150 and IE1 exon 4 segment; and gB, gH, gL, pp65 and pp150; gp64; or portion of such CMV antigens; or p53 or a portion thereof, or a CMV antigen or portion thereof and p53 or a portion thereof; and, such a portion thereof can be an antigenic portion; for instance, an epitope of interest.

It is a yet further object of the invention to provide such methods and compositions in conjunction with additional treatment methods and compositions.

It is another object of the invention to provide

diagnostic methods and compositions.

It is a further object of the invention to provide such diagnostic methods and compositions, including screening a sample from a patient for specific viral proteins or antibodies thereto that predict whether the virus has been reactivated such as IE72, IE84, IE55 and the like; and/or detecting whether CMV nucleic acid such as mRNA is present in PBMCs, e.g., by PCR (such as RT-PCR); and/or detecting whether a cellular-mediated immune response to CMV peptides or proteins is present, e.g., whether PBMCs recognize and/or respond to CMV peptides or proteins, e.g., by administering a CMV skin test by administering CMV proteins or peptides intradermally or subcutaneously or intramuscularly and ascertaining the result of the skin test and/or presenting CMV proteins or peptides to a patient's PBMCs and measuring either the proliferative response of the cells (PMBCs) or the cytokine profile; and/or HLA phenotyping and/or HLA genotyping; and optionally screening a sample from a patient (e.g., sera, blood, SMCs, lesions, etc.) for p53; with optional initial screening for antibodies to CMV, which may optionally be omitted.

It is yet another object of the invention to demonstrate a relation between the presence of antibodies to CMV at the time of angioplasty, indicating prior exposure to CMV, and the subsequent development of restenosis.

It is a still further object of the invention to provide compositions and methods arising as a consequence of demonstrating that there is such a relation.

It is still another object of the invention to show a connection between CMV and restenosis and/or atherosclerosis, especially mechanisms involving the virus, including such as the virus, by inhibiting either the capacity of p53 to block cell cycle progression, or its capacity to initiate apoptosis, enhances SMC accumulation and thereby facilitates development of restenotic lesions.

It is even a still further object of the invention to provide compositions and methods arising as a consequence of demonstrating that there is such a connection and/or mechanisms.

The present invention thus provides methods and compositions for the diagnosis of, prophylaxis of and/or therapy for restenosis and/or atherosclerosis.

The present invention further provides such methods and compositions for prophylaxis and/or therapy which comprise compositions for decreasing viral burden, e.g., vaccine or immunological compositions.

The present invention also provides such methods and compositions including gene products from *in vitro* and/or *in vivo* expression from plasmid DNA, a vector system, such as a recombinant viral or DNA expression system.

The present invention additionally provides such methods and compositions wherein the gene products comprise a CMV antigen, e.g., IE1 and/or IE2, or other viral gene products or portion thereof, or p53 or a portion thereof, or a CMV antigen or portion thereof and p53 or a portion thereof; and, such a portion thereof can be an antigenic portion; for instance, an epitope of interest.

The present invention even further provides such methods and compositions in conjunction with additional treatment methods and compositions.

The present invention thus provides an immunological composition, preferably a vaccine, against cytomegalovirus and/or p53 for therapy for restenosis and/or atherosclerosis; and, a method for providing therapy for restenosis and/or atherosclerosis comprising administering the immunological composition or vaccine against cytomegalovirus (CMV) and/or p53.

The CMV antigen can be IE1 or an antigenic portion thereof, IE2 or an antigenic portion thereof, or, or another CMV gene product or an antigenic portion thereof, wherein the antigenic portion can be an epitope of

interest; and, can be of any suitable origin, e.g., human CMV, murine CMV or rat CMV origin, with human CMV (HCMV) preferred.

The p53 can be wild-type or a mutant, e.g., full-length p53 or a truncated antigenic portion thereof; again, wherein the antigenic portion can be an epitope of interest.

The antigen(s) can be derived recombinantly, e.g., from expression by a virus, bacteria, or plasmid, *in vitro*, with subsequent isolation and purification; or from expression by a recombinant or plasmid *in vivo*. Preferred vector systems include plasmid DNA, adenovirus, baculovirus, poxvirus, and DNA expression systems. For *in vivo* use, plasmid DNA, a recombinant adenovirus or poxvirus, such as a vaccinia virus or avipox virus (e.g., canarypox virus), or a DNA expression system are preferred; but, any suitable vector system, including may be employed. Thus, as herpesvirus vectors are known, a replication-deficient herpesvirus vector, e.g., a replication-defective HSV or CMV vector could even be used in embodiments of the invention.

The invention thus provides compositions and methods for stimulating an immune response, preferably a cellular immune response, directed against CMV and/or p53 to inhibit or prevent restenosis and/or atherosclerosis and/or smooth muscle proliferation. Such a response can cause lysis of infected cells thereby eliminating virus or reducing virus load, and thus inhibit smooth muscle cell proliferation and/or restenosis and/or atherosclerosis. Thus, the invention provides methods and compositions for inducing cell lysis of infected smooth muscle cells and/or inhibition of smooth muscle cell proliferation to treat or prevent restenosis and/or atherosclerosis.

The administration of the immunological composition or vaccine can be after angioplasty, coronary and/or peripheral angioplasty, to prevent the development of, or to provide treatment for, atherosclerosis and/or

restenosis. Thus, the invention provides a therapeutic method for treatment of atherosclerosis and/or restenosis, and compositions therefor.

The immunological composition or vaccine can be administered alone or with additional therapeutic treatment; and, the invention further provides additional methods and compositions for therapeutic treatment of restenosis and/or atherosclerosis.

The additional therapeutic treatment can comprise the administration of: antioxidants which inhibit the cytopathic effect of viral infection, and/or compositions which reduce the transcriptional activity of CMV (transcriptional activity reducer) and/or compositions which decrease reactive oxygen species (ROS) generated by the arachidonic cascade and/or the xanthine/xanthine oxidase system (ROS reducer).

Thus, the invention still further provides to a method for treatment of atherosclerosis and/or restenosis comprising administering a sufficient dose or doses of at least one antioxidant which inhibits the cytopathic effect of viral infection and/or transcriptional activity reducer and/or ROS reducer, either alone, or in conjunction with the aforementioned immunological composition or vaccine therapy; and, the invention provides such compositions.

The antioxidant can be one or more of Vitamin C, Vitamin E, NAC, PDTC, and the like.

The transcriptional activity reducer can be an antiviral drug such as gancyclovir and/or acyclovir (which interfere with viral replication), and/or an antioxidant, or the like.

The ROS reducer can be aspirin (acetylsalicylic acid) or a derivative thereof, ASA, oxypurinol, and the like.

Accordingly, the invention additionally provides a method for treating restenosis and/or atherosclerosis comprising, before, during or after angioplasty, or at any time during the lifetime of the individual, from childhood

to adulthood, to prevent the development or progression of atherosclerosis: administering a sufficient dose or doses of an immunological composition, preferably a vaccine, against CMV and/or p53; or administering a sufficient dose or doses of an immunological composition, preferably a vaccine, against CMV and/or p53 in conjunction with a sufficient dose or doses of at least one antioxidant which inhibits replication and the cytopathic effect of viral infection and/or transcriptional activity reducer and/or ROS reducer; or administering a sufficient dose or doses of at least one antioxidant which inhibits replication and the cytopathic effect of viral infection and/or transcriptional activity reducer and/or ROS reducer. And, the invention provides compositions for these methods.

The compositions are administered before, during, or after angioplasty; before angioplasty, to prevent, i.e., as a prophylaxis against, restenosis and/or atherosclerosis. They can also be administered any time during the lifetime of the individual, from childhood to adulthood, to prevent the development or progression of atherosclerosis.

Accordingly, the invention provides a method for preventing restenosis and/or atherosclerosis comprising, before, during, or after angioplasty to prevent, e.g., as a prophylaxis against restenosis and/or atherosclerosis, or at any time during the lifetime of the individual, from childhood to adulthood, to prevent the development or progression of atherosclerosis: administering a sufficient dose or doses of an immunological composition, preferably a vaccine, against CMV and/or p53; or administering a sufficient dose or doses of an immunological composition, preferably a vaccine, against CMV and/or p53 in conjunction with a sufficient dose or doses of at least one antioxidant which inhibits the cytopathic effect of viral infection and/or transcriptional activity reducer and/or ROS reducer; or administering a sufficient dose or doses of at least one antioxidant which inhibits the cytopathic effect of viral

infection and/or transcriptional activity reducer and/or ROS reducer. And, the invention provides compositions for these methods.

The invention further provides therapeutic or prophylactic compositions and methods of the invention relating to plasmid DNA or recombinants, especially for *in vivo* use, expressing a CMV antigen or portion thereof, or p53 or a portion thereof, or a combination of a CMV antigen or portion thereof and p53 or a portion thereof; and, these recombinants can additionally express or be used in conjunction with another form of molecular based therapy, e.g., expression of cytotoxic molecules to proliferating smooth muscle cells, gene therapy, or antisense strategies to inhibit expression of gene products for cell proliferation. The invention can provide compositions and methods directed at reducing viral load and inhibiting SMC proliferation.

Accordingly in certain aspects, the present invention provides vaccine or immunological compositions for treatment or prophylaxis of restenosis and/or atherosclerosis, including compositions containing a CMV antigen or portion thereof, e.g., IE1, IE2, IE2 and IE2, or antigenic portions thereof, p53 or an antigen portion thereof, a CMV antigen or portion thereof and p53 or portion thereof, such as compositions containing a recombinant or recombinants expressing the CMV antigen or portion thereof and/or p53 or antigenic portion thereof or such an antigen or portion thereof from recombinant expression, uses of such compositions with additional treatment or therapy, including compositions containing a recombinant or recombinants expressing a component of such additional treatment or therapy or co-expressing the component of such additional treatment or therapy with the CMV antigen or portion thereof and/or p53 or antigenic portion thereof, and methods of making and using such compositions (wherein a portion of an antigen can be an epitope of interest).

Recombinants used in the present invention can be of varied type; for instance, one antigen or portion thereof or component of additional therapy may be expressed in one type of system, and another antigen or portion thereof or component of additional therapy (if present) may be from the same, or a different, system.

Plasmid DNA or recombinants of the present invention can have *in vivo* expression at any suitable level for treatment and/or prophylaxis of restenosis and/or atherosclerosis, which can be determined by the skilled artisan without undue experimentation.

Recombinants can be administered in an amount of about 10^7 pfu; thus, the inventive compositions can contain, and the inventive methods involve, administering a composition containing recombinant(s), at least this amount; more preferably about 10^4 pfu to about 10^{10} pfu, e.g., about 10^5 pfu to about 10^9 pfu, for instance about 10^6 pfu to about 10^8 pfu. And, if more than one gene product is expressed by more than one recombinant, each recombinant can be administered in these amounts; or, each recombinant can be administered such that there is, in combination, a sum of recombinants comprising these amounts.

In naked DNA and DNA plasmid compositions, the dosage should be a sufficient amount of naked DNA or DNA plasmid to elicit a response analogous to the expressed antigen compositions; or expression analogous to dosages in expressed antigen compositions; or expression analogous to expression obtained *in vivo* by other, e.g., viral, recombinant compositions. For instance, suitable quantities of naked DNA or plasmid DNA in naked DNA or DNA plasmid compositions can be 1 ug to 100 mg, preferably 0.1 to 10 mg, but lower levels such as 0.1 to 2 mg or even 1-10 ug, may be employed.

And, if more than one gene product is expressed by more than one recombinant and/or DNA (naked or plasmid) system, each recombinant and/or DNA system can be administered in these amounts; or, each recombinant and/or

DNA system can be administered such that there is, in combination, a sum of recombinants and/or DNA comprising these amounts.

Subcutaneous, intradermal or intramuscular administration are presently preferred.

The present invention includes diagnostic methods and compositions.

The present invention also provides such diagnostic methods and compositions, including screening a sample from a patient for specific viral proteins or antibodies thereto that predict whether the virus has been reactivated such as IE72, IE84, IE55 and the like; and/or detecting whether CMV nucleic acid, e.g., mRNA is present in PBMCs, e.g., by PCR (such as reverse transcriptase or RT-PCR); and/or detecting whether a cellular-mediated immune response to CMV peptides or proteins is present, e.g., whether PBMCs recognize and/or respond to CMV peptides or proteins, e.g., by administering a CMV skin test by administering CMV proteins or peptides intradermally or subcutaneously or intramuscularly and ascertaining the result of the skin test and/or presenting CMV proteins or peptides to a patient's PBMCs and measuring either the proliferative response of the cells (PMBCs) or the cytokine profile; and/or HLA phenotyping and/or HLA genotyping; and optionally screening a sample from a patient (e.g., sera, blood, SMCs, lesions, etc.) for p53; with initial screening for antibodies to CMV or proteins from CMV, which may optionally be omitted.

The diagnostic method of the invention can also be used to test for stratification of atherosclerosis and/or restenosis risk factors.

The present invention includes demonstrating a relation between the presence of antibodies to CMV at the time of angioplasty, indicating prior exposure to CMV, and the subsequent development of restenosis.

The present invention also provides compositions and methods arising as a consequence of demonstrating that

there is such a relation.

The present invention includes a showing of a connection between CMV and restenosis and/or atherosclerosis, especially mechanisms involving the virus, including such as the virus, by inhibiting either the capacity of p53 to block cell cycle progression, or its capacity to initiate apoptosis, enhances SMC accumulation and thereby facilitates development of restenotic lesions.

The present invention additionally provides compositions and methods arising as a consequence of demonstrating that there is such a connection and/or mechanisms.

The invention further comprehends methods for preparing the compositions of the invention.

These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF FIGURES

The following Detailed Description, given by way of example, but not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying Figures, incorporated herein by reference, in which:

Figure 1 shows the influence of prior HCMV infection on cumulative distribution of percent stenosis of target vessels determined by angiography 6 months following DCA (Eighty-five target vessels from 75 patients were divided into two groups based on anti-CMV IgG antibody seropositivity status at study entry. A positive CMV IgG antibody status was defined, prospectively, as a cytomegalisa value of ≥ 0.25. Vessels from seropositive patients had higher percent stenoses compared with those from seronegative patients (p=0.01));

Figure 1A shows the incidence of restenosis (>50% diameter narrowing) in the seropositive/seronegative patients;

Figure 2 shows the cumulative percent

distribution of MLD at base line, immediately after the DCA procedure, and at six-month follow-up (See text and Table 2 for detailed statistical analysis);

Figure 3 shows the cumulative percent distribution of luminal diameter loss index (The loss index (late loss divided by acute gain) was higher in the seropositive than in the seronegative patients (p=0.0005));

Figure 4 shows the patients' anti-CMV IgG antibody titer status at study entry and six months following the DCA procedure;

Figure 5 shows patterns of anti-CMV IgG antibodies and T lymphocyte proliferation to CMV antigens in healthy individuals (Serum IgG antibodies for CMV were determined using an ELISA kit (CYTOMEGELISA II, Biowhittaker, Walkersville, MD). Antibody titers were calculated from standard curves provided by the manufacturer. The threshold value for a "positive" result was that provided by the company, which we used prospectively: an ELISA value of less than 0.25 units was considered a negative result, and a value of 0.25 unit or higher was considered a positive result, indicating prior exposure to CMV. Samples for anti-CMV IgG antibodies were tested in triplicate and in two separate experiments. T lymphocyte proliferative responses were performed in 96-well flat-bottom plates (Costar, Cambridge, MA). 100 μl of PBMCs (3 x 10^6/ml) was added to each well. PBMCs were cultured at 37°C with 5% CO_2 in RPMI 1640 (Gibco) containing 5% human AB serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and Herpes buffer, with or without exposure to CMV antigens. After 6 days of culture (3 days for PHA stimulation), each well was pulsed with 1 μCi of [^{3}H]thymidine, and harvested 18 hours later. Thymidine incorporation was determined using a model LS1801 ß-spectrometer (Beckman Instruments, Fullerton, CA). All samples were assayed in triplicate and expressed as the mean counts per minute (cpm). The data are presented as stimulation index (cpm of cultures in the presence of CMV

antigens divided by cpm of cultures in the absence of CMV antigens). If a sample had a response to two of the three CMV antigen preparations (heat inactivated supernatants of CMV-infected fibroblasts, CMV-infected cell lysates, or fixed CMV-infected fibroblasts) and the stimulation index in each was above 4.0, the response was considered positive);

Figures 6A to D show the percentage of individuals with HLA-B44, DR7 and B35 in different CMV-induced immune response subgroups (HLA typing was performed on PBMCs by the NIH HLA laboratory. The standard NIH microcytotoxicity method was used for HLA class I and some class II typing (K. A. Hopkins, A. van Leeuwen, G. N. Tardiff, W. M. LeFor, in *ASHI laboratory manual; Lymophotoxicity testing*, Zachary A. A. and G. A. Teresi, Eds., (Lenexa, kansas: American Society for Histocompatibility and immunogenetics, 1990), pp. 195). Most class II types were determined by PCR (F.M. Marincola et al., J. Immunother. **18**, 242 (1995)). Data were analyzed by the chi-square test or Fisher's exact test using the Instat program (GraphPAD Software, San Diego, CA). All tests were two-tailed. P values less than 0.05 were considered significant);

Figure 7 shows HLA-B35 and positive T-cell proliferative response to CMV antigens in CMV-seronegative individuals (The percentage of seronegative individuals with HLA-B35 who developed a T-cell proliferative response to CMV antigens was significantly higher (P=0.02) than the percentage of seronegative individuals without HLA-B35);

Figure 8 shows the nucleotide sequence for RCMVIE1 (DNA) (SEQ ID NO:47);

Figure 9 shows the nucleotide sequence for RCMVIE2 (DNA) (SEQ ID NO:48);

Figure 10A and B show the nucleotide sequence for RCMVIE2 (DNA) (SEQ ID NO:49);

Figure 11 shows the generation of baculovirus and gene expression with the Bac-To-Bac Expression System;

Figure 12 shows the map and restriction sites for the pFastBac HT expression vector;

Figure 13 shows multiple cloning site sequences for the pFastBac HT expression vector;

Figure 14 shows the nucleotide sequence for HCMVIE2 (DNA) (SEQ ID NO:50); and

Figures 15A and B, respectively, show Western Blot and Coomassie Blue stained gel (Figure 15A: lane 1 = SF9 insect cell lysate, lane 2= baculovirus RCMVIE1 infected SF9 cell lysate, lane 3= RCMVIE1 purified protein preparation, lane 4 = baculovirus RCMVIE2 infected SF9 cell lysate, lane 5 = RK-13 cells, lane 6 = vP1479 infected RK-13 cell lysate, lane 7 = prestained molecular weight markers; Figure 15B: lane 1 = RCMVIE1 purified protein preparation, lane 2 = prestained molecular weight markers).

DETAILED DESCRIPTION

As discussed above, the present invention pertains to methods for diagnosis, prophylaxis and treatment of restenosis and/or atherosclerosis, including detecting cellular mediated immune responses and/or HLA phenotyping and/or genotyping, and administering an agent to reduce viral load in a patient in need of such, for instance administering a vaccine or immunological composition against CMV and/or p53. The vaccine or immunological composition can boost the immune response so that the patient's system consequently reduces viral load.

Examples 1 and 2 show the correlation between CMV and vascular disease, and that while there is a correlation between antibodies to CMV and chances of restenosis occurring, diagnostic methods should include detecting cellular mediated immune response and/or HLA phenotyping and/or genotyping, and methods for treatment or prophylaxis can be aimed at decreasing viral load, such as by administering a vaccine or immunological composition against CMV and/or p53.

Example 1, below, may be summarized as follows:

Background: Recent evidence suggests a potential

role of cytomegalovirus (CMV) in the development of restenosis: CMV DNA is present in restenosis lesions from atherectomy specimens, and a CMV immediate early gene protein (IE84) binds to and inhibits p53, a gene product that can block cell cycle progression and initiate apoptosis. These p53-mediated effects may contribute to increased SMC accumulation and thereby predispose to restenosis.

Methods: Seventy-five consecutive patients undergoing directional coronary atherectomy (DCA) for symptomatic CAD were prospectively evaluated by measuring anti-CMV IgG antibodies (before DCA) to determine whether prior CMV exposure increases restenosis risk, as determined by a 6-month post-DCA angiogram.

Results: Following the DCA procedure, minimal luminal diameter was greater in CMV seropositive patients (n=49) than in seronegative patients (3.18 ±.51 mm vs 2.89±.45, P=0.01); at six months, however, the large late luminal diameter loss (1.24 ±.83 mm vs 0.68 ±.69, P=0.003) and loss index (0.68 ±.47 vs 0.36 ±.33, P<0.001) experienced by seropositive patients resulted in a significantly higher rate of restenosis (43% vs 8%, P=0.002). Both CMV seropositivity (odds ratio=12.9) and CMV titer (odds ratio=8.1) were independently predictive of restenosis (>50% narrowing) in a multivariable logistic regression model. There was no evidence of acute infection, as anti-CMV IgG antibody titers did not increase over time and anti-CMV IgM antibodies were negative in all patients.

Conclusions: Prior infection with CMV is a strong independent risk factor for restenosis.

In more detail, Example 1 provides the first prospective evidence indicating that prior exposure to CMV, as indicated by the presence of CMV IgG antibodies at the time of coronary angioplasty, is a strong independent risk factor for the subsequent development of restenosis (p=0.002; Figure 1). The importance of prior exposure to CMV infection as a risk factor is further emphasized by the

odds ratio of developing restenosis, which was 9-fold greater in patients exposed to CMV than those without such exposure (Table 3). In contrast, no significantly increased risk was seen with any of the other variables tested, findings generally consistent with the results of other studies, e.g., Bach et al., Thromb. Res. 1994; 74:S55-S67; Hermans et al., J. Cardiovas. Pharmaco. 1993; 22(suppl.4):S445-S57; Feuvre et al., Am. J. Cardiol. 1994; 73:840-844; Dzavik et al., Am. J. Cardiol. 1995; 75:936-938; Stein et al., Circulation 1995; 91:979-989; Foley et al., Circulation 1994; 1239-1251.

Analyses believed to provide more complete information than the results of the simple dichotomous analysis described above (restenosis vs no restenosis), led to the same conclusion--that CMV is an important risk factor in the development of restenosis. Thus, when the degree of stenosis is considered as a continuous variable and the effects of CMV are assessed, seropositive patients had a greater degree of lesion stenosis ($p=0.01$; Figure 1, Table 2). With MLD considered as a continuous variable (Figure 2, Table 2), Applicants found that lesion MLD was greater immediately post DCA in the seropositive patients ($p=0.01$). However, the CMV seropositive patients experienced a markedly greater late loss ($p=0.003$) and late loss index ($p=0.0005$), resulting in a tendency for a smaller MLD and a significantly greater incidence of restenosis ($p=0.002$).

Given that the processes leading to restenosis are complex and undoubtedly multifactorial, it is all the more compelling that one factor--exposure to CMV--conveys such a high risk. Indeed, it is probably this very potency of CMV as a risk factor that accounts for the significant relation Applicants found between anti-CMV antibodies and the incidence of restenosis despite the moderate patient sample-size studied. Also helping the sensitivity and specificity of the study is the fact that the diagnosis of restenosis in this study was based on angiographic analysis

rather than on clinical assessment, which is known to be highly inaccurate in predicting anatomic restenosis. Confidence in the results also derives from the fact that this study was prospective in design, that angiographic readers were blinded as to patients' anti-CMV antibody status, and that analysis of anti-CMV antibody levels was performed without knowledge of the angiographic results.

The association between the development of restenosis and CMV was based on anti-CMV IgG antibodies drawn at the time of the angioplasty procedure. Antibody levels did not increase over the ensuing months. This finding, in conjunction with the fact that IgM antibodies were not elevated, suggest that acute CMV infection with systemic viremia did not occur. Although Applicants do not rule out the possibility of acute viremia occurring shortly after angioplasty, with antibody levels returning to baseline by the 6 month repeat studies, Applicants' results are most compatible with the concept that the virus produced either an abortive infection (viral gene expression limited to immediate early gene products), or that viral replication occurred locally in the absence of systemic viremia.

CMV is a complex virus--it has a large genome with over 200 open reading frames. Thus, it undoubtedly possesses many viral proteins that might influence neointimal accumulation. In addition to the effects of IE84, which as noted hereinabove binds to and inactivates p53, infection of SMCs with CMV leads to the expression and secretion of growth factors, Gonczol et al., J. Gen. Virol. 1984; 65:1833-1837; Alcami et al., J. Gen. Virol. 1991; 72:2765-2770, and CMV infection has been shown to activate NFκB, Kowalik et al., Proc. Natl. Acad. Sci. USA 1993; 90:1107-1111, a transcription factor involved in stimulating a broad range of genes, including those involved in inflammatory and immune responses. The virus also increases leukocyte and platelet adhesion to endothelial cells through induction of cellular expression

of adhesion molecules, Grundy et al., Immunology. 1993; 78:405-412; O'Brien et al., J. Clin. Invest. 1993; 92:945-951; Span et al., Eur. J. Clin. Invest. 1991; 21:331-338; Etingin et al., Proc. Natl. Acad. Sci. USA 1993; 90:5153-5156; and induces changes that are procoagulant, Van Dam-Mieras et al., Thromb. Haemost. 1992; 68:364-370; Etingin et al., Cell 1990; 61:657-662; Pryzdial et al., Blood 1994; 84:3749-3757. CMV also increases the activity of the scavenger receptor, and IE72, another IE gene product, increases scavenger receptor gene expression, Zhou et al., Circulation 1995; 92:1-162 (Abstr.); increased accumulation of oxidized LDL within lesion SMCs might contribute to an atherogenic-related process like restenosis. Finally, it has recently been shown that IE72 and IE84 inhibit apoptosis, which could increase neointimal accumulation, Zhu et al., J. Virol. 1995; 69:7960-7970.

Totally unexpectedly, Applicants found a strong association between CMV and hypertension. Thus, there may be an important CMV-hypertension link, such that testing for CMV may be indicative of a predisposition to hypertension and *vice versa*.

It is possible, although Applicants do not necessarily wish to be bound by any one particular theory, that the relation Applicants observed between CMV infection and subsequent development of restenosis is due to a specific relation between the particular angioplasty procedure used in the present investigation--atherectomy--and that very different results may be observed with other techniques such as balloon angioplasty. This possibility appears very remote, as it is generally believed that the final common pathway of the restenosis process is a healing response to vascular injury, a response that probably would be similar (and therefore influenced in a similar way by CMV) whether the injury were induced by balloon angioplasty or by directional atherectomy. Moreover, adjunct balloon dilatation was in fact performed in 87% of patients. Thus, the particular angioplasty procedure is believed to not be

a factor.

It is possible that CMV seropositivity, instead of indicating a causal role of CMV per se in restenosis, is just a marker of another process that is actually the mechanistically contributing factor. However, CMV DNA is present in human restenosis, and a CMV gene product inhibits the transcriptional activity of p53 in human coronary artery smooth muscle cells, Speir et al., Science 1994; 265:391-394, and acute CMV infection increases neointimal formation in a rat balloon injury model, Zhou et al., J. Am. Cell. Cardiol. 1995; (suppl) 242a (Abstr.), which when taken together with the results presented herein, strongly suggest that CMV does indeed play a role in restenosis development. (However, the Abstract of Zhou et al., *supra*, either individually or in a combination with other documents, cannot be said to teach or suggest the present invention because, in addition to the surprising results in the Examples, Zhou et al., *supra* concerns an acute infection model, whereas human or animal patients are chronically infected).

The results of the present invention demonstrate that CMV seropositivity provides a powerful means of risk-stratifying patients for the development of restenosis. Thus, the determination (from a simple, standard blood test) that a given patient has less than a 10% chance of developing restenosis (CMV seronegative) vs over a 40% chance (CMV seropositive), when considered together with the patient's specific clinical profile, could importantly influence the clinician's decision as to whether that patient might best benefit from bypass surgery or from angioplasty.

However, as shown by Example 2, the CMV seropositive or seronegative status of a patient, while providing particular statistical chances of developing restenosis (Example 1), is not necessarily in and of itself sufficient in providing a diagnosis as to whether there is a predisposition towards or against (prevention of)

restenosis and/or atherosclerosis; but rather, detecting a patient's cell mediated immune response to CMV and/or HLA phenotyping and/or genotyping may be more predictive of such a predisposition.

More particularly, because the type of immune response (cellular vs humoral) to infectious agents can determine disease expression or containment, and because cytomegalovirus (CMV) may contribute to restenosis and atherosclerosis, as reported in Example 2, Applicants tested whether there is a spectrum of humoral vs cellular immunodominant responses to CMV infection in healthy individuals. Four patterns were found: both cellular and humoral; humoral only; no detectable response; and, unexpectedly, cellular only. Applicants then determined whether HLA phenotype influenced the type of response: 50% of individuals with a cellular, but not humoral, immunodominant response had an HLA-B35 allele without HLA-B44; conversely, 43% with a humoral, but not cellular, immunodominant response had HLA-B44 without HLA-B35. These values significantly differed from those of control populations. Thus, genetically-determined, HLA-associated, immunodominant patterns of response to CMV occur and may influence susceptibility to CMV-induced disease, including vascular disease.

Pathogen-induced activation of the cellular and the humoral arms of the immune system are frequently inversely related. This observation has led to important insights relating to the type of immune response (cellular or humoral) that permits some hosts either to succeed in eliminating potential pathogens, or to develop persistence of pathogen and the establishment of chronic or recurrent disease.

Although the humoral arm of the immune system is important mainly for prevention of infection by extracelluar agents, if pathogens gain entry to intracellular sites, the cell-mediated immune response becomes essential to pathogen elimination or control.

There is now evidence indicating that the cell-mediated immune response is an important mechanism for eliminating or controlling infectious pathogens that cause chronic disease in humans and in various animal species. Data compatible with this concept come from studies of infectious diseases such as acquired immune deficiency syndrome (AIDS) (S. Rowland-Jones et al., Nat. Med. **1**, 59 (1995); M. Clerici, JAMA. **271**, 42 (1994)), chronic hepatitis B (B. Rehermann, D. Lau, J. H. Hoofnagle, F. V. Chisari, J. Clin. Invest. **97**, 1655 (1996)), and leishmaniasis (S. C. Mendonca, P. M. De Luca, W. Mayrink, T. G. Restom, Am. J. Trop. Med. Hyg. **53**, 195 (1995); M. L. Guler et al., Science **271**, 984 (1996); N. Noben-Trauth, P. Kropf, I. Muller, Science **271**, 987 (1996)). On the other hand, a chronic cell-mediated immune inflammatory response can also lead to disease exacerbation.

Given, as shown in Example 2, that the same HLA molecule that predisposes to a cellular immunodominant response to CMV is also associated with a cellular immune response targeted to HIV and to the *P. falciparium* parasite (which seems to convey a protective effect in these diseases), these results herein have much broader implications.

Specific HLA molecules, such as HLA-B35, may have unique attributes that facilitate the development of a cellular immunodominant response, implying a mechanism whereby some individuals are resistant to certain infectious diseases (or to cancer), and some are susceptible to the development of diseases characterized by immunopathology (chronic granulomatous diseases and autoimmune disease).

There may be a correlation between this pattern of immune response and either protection from, or exacerbation of, any disease processes caused by CMV, including vascular disease.

Thus, novel therapeutic strategies, such as disclosed herein arise. For instance, the results reported

herein allow for favorably altering disease outcome by directing attempts to change the immunodominant phenotype from one that increases disease susceptibility to one that promotes resistance.

More importantly, Example 2 shows that diagnosis for a predisposition towards restenosis from angioplasty or for a predisposition towards atherosclerosis cannot be predicated on merely whether an individual has antibodies against CMV, i.e., any prior correlations between CMV and vascular disease fail to teach or suggest the methods and compositions for diagnosis and therapy or treatment or prophylaxis of the present invention.

For instance, Example 2 demonstrates that detecting cellular immune responses and/or HLA genotyping and/or phenotyping can provide surprisingly better diagnosis. Detection of a cellular mediated response can be more predictive or predisposition to or against (prevention) of restenosis and/or atherosclerosis, since antibody-negative patients, as herein demonstrated can have T-cell responses.

Further, this Examples 1 and 2 show the importance in therapy or treatment or prophylaxis to boost the immune response to CMV and/or p53. Simply, the latent CMV infection is a low grade viral infection that the body cannot rid itself of because there is not sufficient stimulation of immune responses. Therapy, treatment or prophylaxis with a vaccine or immunological composition against CMV and/or p53 can thus boost the immune response to eliminate low levels of CMV, e.g., to reduce activation, and thus provide therapy, treatment or prophylaxis with respect to restenosis and/or atherosclerosis.

And, with the now disclosed causal role of CMV in the development of restenosis, and the showing that measuring antibodies against CMV is not sufficient for predicting predisposition towards or against restenosis and/or atherosclerosis, the therapeutic approaches to the prevention and/or treatment of restenosis and/or

atherosclerosis, as herein disclosed, e.g., immunological or vaccine compositions comprising CMV antigens or portions thereof and/or p53 or portions thereof, or such compositions in conjunction with additional therapies or treatments, and methods employing them, as well as the diagnostic methods including detecting cell mediated immune response and/or HLA phenotyping and/or genotyping, are now provided.

Thus, in a general way, the invention provides a composition comprising a CMV antigen or antigens, or portions thereof and/or p53 or a portion thereof, and methods for making and using the composition in treatment, therapy or prophylaxis of restenosis and/or atherosclerosis. The composition can be a vaccine or immunological composition. The antigen(s) and/or p53 or portions thereof can be from *in vitro* and/or *in vivo* expression by a plasmid, a recombinant, or from isolation and/or purification from cells expressing the antigen(s) and/or p53, e.g., cells infected with HCMV and subsequent isolation and/or purification.

Techniques for protein purification of native proteins, in general, are as follows:

Briefly, the cells are disrupted and the protein of interest is released into an aqueous "extract". There are many methods of cellular disintegration, which vary from relatively gentle to vigorous conditions, and the choice of one method over the other is dependent upon the source material. Animal tissues vary from the very easily broken erythrocytes to tough collagenous material such as found in blood vessels and other smooth-muscle containing tissue. Bacteria vary from fairly fragile organisms that can be broken up by digestive enzymes or osmotic shock to more resilient species with thick cell walls, needing vigorous mechanical treatment for disintegration.

Gentle techniques include cell lysis, enzymatic digestion, chemical solubilization, hand homogenization and mincing (or grinding); moderate techniques of cell

disintegration include blade homogenization and grinding with abrasive materials, i.e., sand or alumina; and vigorous techniques include french press, ultrasonication, bead mill or Manton-Gaulin homogenization. Each of the aforementioned techniques are art-recognized, and it is well within the scope of knowledge of the skilled artisan to determine the appropriate method of cell disintegration based upon the starting material, and the teachings herein and in the art.

Following cell disintegration, the extract is prepared by centrifuging off insoluble material. At this stage, one may proceed with the purification method, as an extract containing as much of the protein of interest as possible has been prepared, and, where appropriate, particulate and most nonprotein materials have been removed.

Standard techniques of protein purification may be employed to further purify the protein of interest, including: precipitation by taking advantage of the solubility of the protein of interest at varying salt concentrations, precipitation with organic solvents, polymers and other materials, affinity precipitation and selective denaturation; column chromatography, including high performance liquid chromatography (HPLC), ion-exchange, affinity, immuno affinity or dye-ligand chromatography; immunoprecipitation and the use of gel filtration, electrophoretic methods, ultrafiltration and isoelectric focusing. Each of the above-identified methods are well within the knowledge of the skilled artisan, and no undue experimentation is required to purify the native proteins or epitopes of interest of CMV or p53, using the standard methodologies outlined hereinabove, and in the literature, as well as the teachings in the Examples below.

In regard to isolation and/or purification of CMV antigen(s) and/or p53 from cells expressing the antigen(s) and/or p53, in addition to methods discussed in the Examples, mention is made of U.S. Patents Nos. 4,689,225

(HCMV gA subunit vaccine), 5,180,813 (early envelope glycoprotein and monoclonals to HCMV glycoproteins), and 4,716,104 (detection of HCMV antigens by antibodies reactive to IE of HCMV). The compositions and methods of these patents may be useful in the practice of the present invention.

Accordingly, the composition can comprise a vector comprising exogenous DNA encoding at least one CMV and/or p53 epitope. The epitope can be: IE1 and/or IE2 or a portion thereof; gB; gB with transmembrane deleted therefrom; gH; gL; pp150; pp65; IE1 with amino acids 2-32 deleted therefrom; IE1 with amino acids 292-319 deleted therefrom; IE1 exon 4 segment; gB and gH; gB and pp65; gB, gH and pp65; gB, gH, pp65 and IE1 exon 4 segment; gB, gH, pp65, pp150, and IE1 exon 4 segment; gB, gH, pp65 and pp150; gB, gH, gL, pp65, pp150 and IE1 exon 4 segment; and gB, gH, gL, pp65 and pp150; or portion of such CMV antigens; and/or p53, wild-type or mutant, or a portion thereof; or, more generally, a CMV antigen or portion thereof and/or p53 or a portion thereof; and, such a portion thereof can be an antigenic portion; for instance, an epitope of interest. The vector preferably induces an immune response, more preferably a protective immune response, when administered to a patient. Mention is made of U.S. Patents Nos. 5,047,320 and 5,075,213, incorporated herein by reference, which relate to DNA probes for HCMV gp64 and HCMV gp64 as a vaccine, such that if desired, an epitope of interest in a composition of the invention can be gp64 or a portion thereof.

The methods for making a vector or recombinant can be by or analogous to the methods disclosed in U.S. Patent Nos. 4,603,112, 4,769,330, 5,174,993, 5,505,941, 5,338,683, 5,494,807, 4,722,848, WO 94/16716, U.S. application Serial No. 08/184,009, filed January 19, 1994, WO 96/39491, U.S. application Serial No. 08/658,665, filed June 5, 1996, Paoletti, "Applications of pox virus vectors to vaccination: An update," PNAS USA 93:11349-11353,

October 1996, Moss, "Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety," PNAS USA 93:11341-11348, October 1996, Smith et al., U.S. Patent No. 4,745,051 (recombinant baculovirus), Richardson, C.D. (Editor), Methods in Molecular Biology 39, "Baculovirus Expression Protocols" (1995 Humana Press Inc.), Smith et al., "Production of Huma Beta Interferon in Insect Cells Infected with a Baculovirus Expression Vector," Molecular and Cellular Biology, Dec., 1983, Vol. 3, No. 12, p. 2156-2165; Pennock et al., "Strong and Regulated Expression of *Escherichia coli* B-Galactosidase in Infect Cells with a Baculovirus vector," Molecular and Cellular Biology Mar. 1984, Vol. 4, No. 3, p. 399-406; EPA 0 370 573, U.S. application Serial No. 920,197, filed October 16, 1986, EP Patent publication No. 265785, U.S. Patent No. 4,769,331 (recombinant herpesvirus), Roizman, "The function of herpes simplex virus genes: A primer for genetic engineering of novel vectors," PNAS USA 93:11307-11312, October 1996, Andreansky et al., "The application of genetically engineered herpes simplex viruses to the treatment of experimental brain tumors," PNAS USA 93:11313-11318, October 1996, Robertson et al. "Epstein-Barr virus vectors for gene delivery to B lymphocytes," PNAS USA 93:11334-11340, October 1996, Frolov et al., "Alphavirus-based expression vectors: Strategies and applications," PNAS USA 93:11371-11377, October 1996, Kitson et al., J. Virol. 65, 3068-3075, 1991; U.S. Patent Nos. 5,591,439, 5,552,143 (recombinant adenovirus expressing HCMV gB and IE-exon 4), Grunhaus et al., 1992, "Adenovirus as cloning vectors," Seminars in Virology (Vol. 3) p. 237-52, 1993, Ballay et al. EMBO Journal, vol. 4, p. 3861-65, Graham, Tibtech 8, 85-87, April, 1990, Prevec et al., J. Gen Virol. 70, 429-434, PCT WO91/11525, Felgner et al. (1994), J. Biol. Chem. 269, 2550-2561, Science, 259:1745-49, 1993 and McClements et al., "Immunization with DNA vaccines encoding glycoprotein D or glycoprotein B, alone or in combination, induces protective immunity in animal models of herpes

simplex virus-2 disease," PNAS USA 93:11414-11420, October 1996, and U.S. Patents Nos 5,591,639, 5,589,466, and 5,580,859 relating to DNA expression vectors, *inter alia*.

Recombinant poxviruses can be constructed in two steps known in the art and analogous to the methods for creating synthetic recombinants of poxviruses such as the vaccinia virus and avipox virus described in U.S. Patent Nos. 4,769,330, 4,772,848, 4,603,112, 5,110,587, 5,179,993, 5,505,941, and 5,494,807, the disclosures of which, like the disclosures of all documents cited herein, are incorporated herein by reference.

First, the DNA gene sequence to be inserted into the virus, e.g., an open reading frame from a non-pox source, is placed into a plasmid construct such as an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the DNA gene sequence to be inserted can be ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA; for instance, pox DNA containing a nonessential locus (although an essential locus may also be used). The resulting plasmid construct is then amplified, e.g., by growth within *E. coli* bacteria (Clewell, 1972) and isolated (Clewell et al., 1969; Maniatis et al., 1982). Alternatively, the DNA gene sequence can, without separate ligation to a promoter, merely be placed within the plasmid construct so that the DNA gene sequence is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA; for instance, a region downstream from an endogenous promoter such that expression of the gene sequence is under control of the promoter and the promoter and coding portion of the DNA gene sequence are thus adjacent.

Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, e.g. chick embryo fibroblasts, along with the

poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively gives a poxvirus modified by the presence, e.g., in a nonessential region of its genome, of foreign DNA sequences. The term "foreign" DNA designates exogenous DNA, particularly DNA from a non-pox source, that codes for gene products not ordinarily produced by the genome into which the exogenous DNA is placed.

However, the foregoing is not meant to limit the vectors or recombinants or means for obtaining vectors or recombinants in the present invention, as any vector or recombinant as well as any means for obtaining a vector or recombinant, e.g. a poxvirus-CMV and/or p53 epitope of interest recombinant, may be used to obtain the present invention.

In some embodiments, a poxvirus vector may be desired.

Paoletti, U.S. Patent No. 5,338,683, incorporated herein by reference, provides poxvirus-herpesvirus recombinants, including vaccinia and avipox virus-herpesvirus recombinants, such as vaccinia and avipox virus-CMV recombinants, and gene products therefrom, useful in the practice of this invention.

A preferred vaccinia vector can have attenuated virulence, such as the NYVAC vector. Preferred avipox vectors include ALVAC (attenuated canarypox virus) and TROVAC (attenuated fowlpox virus). ALVAC and TROVAC are each unimolar species.

ALVAC has been deposited with the ATCC, Accession No. VR-2547, under the terms of the Budapest Treaty. ALVAC is an attenuated canarypox virus-based vector that was a plaque-cloned derivative of the licensed canarypox vaccine, Kanapox (Tartaglia et al., 1992). ALVAC has some general properties which are the same as some general properties of Kanapox.

ALVAC-based recombinant viruses expressing extrinsic immunogens have also been demonstrated

efficacious as vaccine vectors (Tartaglia et al., 1993a,b). So too have NYVAC-based recombinant viruses expressing extrinsic immunogens. In Paoletti et al., U.S. Patent No. 5,494,807, incorporated herein by reference, ALVAC-HCMV and NYVAC-HCMV recombinants, e.g., such recombinants expressing HCMV gB, which elicit neutralizing antibodies, cell mediated immunity, and epitope-specific cytotoxic T-lymphocytes, and gene products therefrom, useful in the practice of this invention, are disclosed.

Paoletti et al., PCT publication WO 96/39491, based on U.S. applications Serial Nos. 08/471,014, filed June 6, 1995, and 08/658,665, filed June 5, 1995, incorporated herein by reference, provides recombinant poxvirus-cytomegalovirus compositions and uses, including NYVAC and ALVAC recombinants, e.g., wherein the exogenous DNA codes for an HCMV protein selected from the group consisting of: gB; gB with transmembrane deleted therefrom; gH; gL; pp150; pp65; IE1; IE1 with amino acids 2-32 deleted therefrom; IE1 with amino acids 292-319 deleted therefrom; IE1 exon 4 segment; gB and gH; gB and pp65; gB, gH and pp65; gB, gH, pp65 and IE1 exon 4 segment; gB, gH, pp65, pp150, and IE1 exon 4 segment; gB, gH, pp65 and pp150; gB, gH, gL, pp65, pp150 and IE1 exon 4 segment; and gB, gH, gL, pp65 and pp150, and gene products therefrom, useful in the practice of this invention.

Paoletti et al. WO 94/16716 based on U.S. applications Serial Nos. 007,115, filed January 21, 1993, and 184,009, filed January 19, 1994, incorporated herein by reference, provides recombinant viruses containing DNA encoding a cytokine and/or tumor associated antigen, including p53, wild-type or mutant, e.g., a NYVAC or ALVAC recombinant containing DNA coding for p53, wildtype or mutant, useful in the practice of this invention.

From the aforementioned Paoletti patent publications, and the teachings herein, including documents incorporated by reference into this specification, the skilled artisan can construct any desired poxvirus-HCMV

and/or p53 recombinant expressing an epitope of interest, without undue experimentation.

Baculovirus, adenovirus, and DNA expression systems are also preferred for the practice of the invention.

With respect to certain vectors or recombinants, such as those whose DNA is infectious, e.g., adenovirus vectors, herpesvirus vectors, and the like, methods analogous to the above-described *in vivo* recombination technique for poxviruses may be employed for construction of the vector or recombinant containing desired exogenous DNA; but, such recombinants or vectors, with reference to adenovirus only for exemplification, may also be obtained by cleaving adenovirus DNA to obtain cleaved adenovirus DNA, ligating the exogenous DNA to the cleaved adenovirus DNA to obtain hybrid adenovirus-exogenous DNA, tranfecting a cell with the hybrid adenovirus-exogenous DNA, and optionally then recovering adenovirus modified by the presence of the exogenous DNA.

U.S. Patents Nos. 5,591,439 and 5,552,143, incorporated herein by reference, provide adenovirus-HCMV gB or IE-exon 4 recombinants and gene products therefrom, useful in the practice of this invention. Furthermore, by employing the techniques of these patents, or of other literature concerning adenovirus recombinants, with exogenous DNA of any of U.S. Patents Nos. 5,047,320, 5,075,213, Paoletti, U.S. Patent No. 5,338,683, Paoletti et al., U.S. Patent No. 5,494,807, Paoletti et al., PCT publication WO 96/39491, based on U.S. applications Serial Nos. 08/471,014, filed June 6, 1995, and 08/658,665, filed June 5, 1995, Paoletti et al. WO 94/16716 based on U.S. applications Serial Nos. 007,115, filed January 21, 1993, and 184,009, filed January 19, 1994, or other documents cited and incorporated herein, or literature concerning HCMV antigens, epitopes of interest, p53, p53 epitopes of interest, and DNA coding therefor, and the teachings herein, adenovirus embodiments expressing any desired HCMV

and/or p53 epitope of interest and obtaining gene products therefrom, are within the ambit of the skilled artisan, without undue experimentation, for practice of this invention.

By employing the techniques of Smith et al., U.S. Patent No. 4,745,051, incorporated herein by reference, or of other literature concerning baculovirus recombinants, with exogenous DNA of any of U.S. Patents Nos. 5,047,320, 5,075,213, Paoletti, U.S. Patent No. 5,338,683, Paoletti et al., U.S. Patent No. 5,494,807, Paoletti et al., PCT publication WO 96/39491, based on U.S. applications Serial Nos. 08/471,014, filed June 6, 1995, and 08/658,665, filed June 5, 1995, Paoletti et al. WO 94/16716 based on U.S. applications Serial Nos. 007,115, filed January 21, 1993, and 184,009, filed January 19, 1994, or other documents cited and incorporated herein, or literature concerning HCMV antigens, epitopes of interest, p53, p53 epitopes of interest, and DNA coding therefor, and teachings herein, baculovirus embodiments expressing any desired HCMV and/or p53 epitope of interest and obtaining gene products therefrom, are within the ambit of the skilled artisan, without undue experimentation, for practice of this invention.

By employing the techniques of U.S. Patents Nos. 5,591,639, 5,589,466, 5,580,589, incorporated herein by reference, or of other literature concerning DNA expression vectors with exogenous DNA of any of U.S. Patents Nos. 5,047,320, 5,075,213, Paoletti, U.S. Patent No. 5,338,683, Paoletti et al., U.S. Patent No. 5,494,807, Paoletti et al., PCT publication WO 96/39491, based on U.S. applications Serial Nos. 08/471,014, filed June 6, 1995, and 08/658,665, filed June 5, 1995, Paoletti et al. WO 94/16716 based on U.S. applications Serial Nos. 007,115, filed January 21, 1993, and 184,009, filed January 19, 1994, or other documents cited and incorporated herein or literature concerning HCMV antigens, epitopes of interest, p53, p53 epitopes of interest, and DNA coding therefor, and

the teachings herein, DNA expression vector embodiments expressing any desired HCMV and/or p53 epitope of interest and obtaining gene products therefrom, are within the ambit of the skilled artisan, without undue experimentation, for practice of this invention.

Similarly, any other desired vector or recombinant expressing any desired HCMV and/or p53 epitope of interest and obtaining gene products therefrom, are within the ambit of the skilled artisan, without undue experimentation, from this disclosure and the knowledge in the art, for practice of this invention.

The expression product generated by vectors or recombinants in this invention can also be isolated from infected or transfected cells and used to inoculate patients in a subunit vaccine configuration (composition, or an antigenic or immunological composition).

Further, DNA encoding a CMV and/or p53 epitope(s) of interest can be administered through immunization using alternate appropriately engineered mammalian expression systems including but not limited to other poxviruses, herpesviruses, adenoviruses, alphavirus-based strategies, and naked or formulated DNA-based immunogens. Techniques for engineering such recombinant subunits are known in the art. With respect to techniques for these immunization vehicles and state-of-the-art knowledge mention is particularly made of: Hormaeche and Kahn, Perkus and Paoletti, Shiver et al. all in Concepts in Vaccine Development, Kaufman, S.H.E., ed., Walter deGruytes, New York, 1996, and vectors described in Viruses in Human Gene Therapy, Vos, J.-M.H., ed, Chapman and Hall, Carolina Academic Press, New York, 1995, and in Recombinant Vectors in Vaccine Development, Brown, F., ed., Karger, New York, 1994.

The invention still further provides an antigenic, immunogenic, immunological or vaccine composition for use in therapy, treatment and/or prophylaxis of restenosis and/or atherosclerosis containing

the recombinant virus or expression product thereof, and an acceptable carrier or diluent. An immunological composition containing the vector or recombinant virus (or an expression product thereof) elicits an immunological response - local or systemic. The response can, but need not be, protective. An immunogenic composition containing the vector or recombinant virus (or an expression product thereof) likewise elicits a local or systemic immunological response which can, but need not be, protective. An antigenic composition similarly elicits a local or systemic immunological response which can, but need not be, protective. A vaccine composition elicits a local or systemic protective response. Accordingly, the terms "immunological composition", "antigenic composition" and "immunogenic composition" include a "vaccine composition" (as the three former terms can be protective compositions). A protective response is understood to be a response, such as a humoral and/or secretory and/or cell-mediated response which confers an immunity, with immunity understood to comprise the ability to resist or overcome infection or to overcome infection more easily as compared to a subject not administered the inventive composition, or to better tolerate infection as compared to a subject not administered the inventive composition, e.g., increased resistance to infection.

As to epitopes of interest, one skilled in the art can determine an epitope or immunodominant region of a peptide or polypeptide and ergo the coding DNA therefor from the knowledge of the amino acid and corresponding DNA sequences of the peptide or polypeptide, as well as from the nature of particular amino acids (e.g., size, charge, etc.) and the codon dictionary, without undue experimentation.

A general method for determining which portions of a protein to use in an immunological composition focuses on the size and sequence of the antigen of interest. "In general, large proteins, because they have more potential

determinants are better antigens than small ones. The more foreign an antigen, that is the less similar to self configurations which induce tolerance, the more effective it is in provoking an immune response." Ivan Roitt, Essential Immunology, 1988.

As to size: the skilled artisan can maximize the size of the protein encoded by the DNA sequence to be inserted into the mammalian vector (keeping in mind the insertion limitations of the vector). To minimize the DNA inserted while maximizing the size of the protein expressed, the DNA sequence can exclude introns (regions of a gene which are transcribed but which are subsequently excised from the primary RNA transcript).

At a minimum, the DNA sequence can code for a peptide at least 8 or 9 amino acids long. This is the minimum length that a peptide needs to be in order to stimulate a CD4+ T cell response (which recognizes virus infected cells or cancerous cells). A minimum peptide length of 13 to 25 amino acids is useful to stimulate a CD8+ T cell response (which recognizes special antigen presenting cells which have engulfed the pathogen). See Kendrew, *The Encyclopedia of Molecular Biology* (Blackwell Science Ltd 1995). However, as these are minimum lengths, these peptides are likely to generate an immunological response, i.e., an antibody or T cell response; but, for a protective response (as from a vaccine composition), a longer peptide is preferred.

With respect to the sequence, the DNA sequence preferably encodes at least regions of the peptide that generate an antibody response or a T cell response. One method to determine T and B cell epitopes involves epitope mapping. The protein of interest "is fragmented into overlapping peptides with proteolytic enzymes. The individual peptides are then tested for their ability to bind to an antibody elicited by the native protein or to induce T cell or B cell activation. This approach has been particularly useful in mapping T-cell epitopes since the T

cell recognizes short linear peptides complexed with MHC molecules. The method is less effective for determining B-cell epitopes" since B cell epitopes are often not linear amino acid sequence but rather result from the tertiary structure of the folded three dimensional protein. Janis Kuby, Immunology, pp. 79-80 (1992).

Another method for determining an epitope of interest is to choose the regions of the protein that are hydrophilic. Hydrophilic residues are often on the surface of the protein and are therefore often the regions of the protein which are accessible to the antibody. Janis Kuby, Immunology, p. 81 (1992).

Yet another method for determining an epitope of interest is to perform an X-ray crystallographic analysis of the antigen (full length)-antibody complex. Janis Kuby, Immunology, p. 80 (1992).

Still another method for choosing an epitope of interest which can generate a T cell response is to identify from the protein sequence potential HLA anchor binding motifs which are peptide sequences which are known to be likely to bind to the MHC molecule.

The peptide which is a putative epitope of interest, to generate a T cell response, should be presented in a MHC complex. The peptide preferably contains appropriate anchor motifs for binding to the MHC molecules, and should bind with high enough affinity to generate an immune response. Factors which can be considered are: the HLA type of the patient expected to be immunized, the sequence of the protein, the presence of appropriate anchor motifs and the occurrence of the peptide sequence in other vital cells.

An immune response is generated, in general, as follows: T cells recognize proteins only when the protein has been cleaved into smaller peptides and is presented in a complex called the "major histocompatibility complex MHC" located on another cell's surface. There are two classes of MHC complexes - class I and class II, and each class is

made up of many different alleles. Different patients have different types of MHC complex alleles; they are said to have a "different HLA type".

Class I MHC complexes are found on virtually every cell and present peptides from proteins produced inside the cell. Thus, Class I MHC complexes are useful for killing cells which when infected by viruses or which have become cancerous and as the result of expression of an oncogene. T cells which have a protein called CD4 on their surface, bind to the MHC class I cells and secrete lymphokines. The lymphokines stimulate a response; cells arrive and kill the viral infected cell.

Class II MHC complexes are found only on antigen-presenting cells and are used to present peptides from circulating pathogens which have been endocytosed by the antigen- presenting cells. T cells which have a protein called CD8 bind to the MHC class I cells and kill the cell by exocytosis of lytic granules.

Some guidelines in determining whether a protein contains epitopes of interest which will stimulate a T cell response, include: Peptide length - the peptide should be at least 8 or 9 amino acids long to fit into the MHC class I complex and at least 13-25 amino acids long to fit into a class II MCH complex. This length is a minimum for the peptide to bind to the MHC complex. It is preferred for the peptides to be longer than these lengths because cells may cut the expressed peptides. The peptide should contain an appropriate anchor motif which will enable it to bind to the various class I or class II molecules with high enough specificity to generate an immune response (See Bocchia, M. et al., Specific Binding of Leukemia Oncogene Fusion Protein Peptides to HLA Class I Molecules, Blood 85:2680-2684; Englehard, VH, Structure of peptides associated with class I and class II MHC molecules Ann. Rev. Immunol. 12:181 (1994)). This can be done, without undue experimentation, by comparing the sequence of the protein of interest with published structures of peptides

associated with the MHC molecules. Protein epitopes recognized by T cell receptors are peptides generated by enzymatic degradation of the protein molecule and are presented on the cell surface in association with class I or class II MHC molecules.

Further, the skilled artisan can ascertain an epitope of interest by comparing the protein sequence with sequences listed in the protein data base. Regions of the protein which share little or no homology are better choices for being an epitope of that protein and are therefore useful in a vaccine or immunological composition. Regions which share great homology with widely found sequences present in vital cells should be avoided.

Even further, another method is simply to generate or express portions of a protein of interest, generate monoclonal antibodies to those portions of the protein of interest, and then ascertain whether those antibodies inhibit growth *in vitro* of the pathogen from which the the protein was derived. The skilled artisan can use the other guidelines set forth in this disclosure and in the art for generating or expressing portions of a protein of interest for analysis as to whether antibodies thereto inhibit growth *in vitro*.

For example, the skilled artisan can generate portions of a protein of interest by: selecting 8 to 9 or 13 to 25 amino acid length portions of the protein, selecting hydrophilic regions, selecting portions shown to bind from X-ray data of the antigen (full length)-antibody complex, selecting regions which differ in sequence from other proteins, selecting potential HLA anchor binding motifs, or any combination of these methods or other methods known in the art.

Epitopes recognized by antibodies are expressed on the surface of a protein. To determine the regions of a protein most likely to stimulate an antibody response one skilled in the art can preferably perform an epitope map, using the general methods described above, or other mapping

methods known in the art.

As can be seen from the foregoing, without undue experimentation, from this disclosure and the knowledge in the art, the skilled artisan can ascertain the amino acid and corresponding DNA sequence of a CMV and/or p53 epitope of interest for obtaining a T cell, B cell and/or antibody response. In addition, reference is made to Gefter et al., U.S. Patent No. 5,019,384, issued May 28, 1991, and the documents it cites, incorporated herein by reference (Note especially the "Relevant Literature" section of this patent, and column 13 of this patent which discloses that: "A large number of epitopes have been defined for a wide variety of organisms of interest. Of particular interest are those epitopes to which neutralizing antibodies are directed. Disclosures of such epitopes are in many of the references cited in the Relevant Literature section.")

The administration procedure for the vector or recombinant or expression product thereof in the invention, and of compositions of the invention such as immunological, antigenic or vaccine compositions which are prophylactic and/or therapeutic compositions with respect to vascular disease, e.g., atherosclerosis and/or restenosis, can be via a parenteral route (intradermal, intramuscular or subcutaneous). Such an administration enables a systemic immune response. The administration can be via a mucosal route, e.g., oral, nasal, genital, etc. Such an administration enables a local immune response. Direct administration to blood vessels and SMCs (see, e.g., Epstein et al., JACC Vol. 23, No. 6, 1994:1278-88 (and documents cited therein, incorporated herein by reference); Chang et al., Science 267:518-22 (January 27, 1995) (and documents cited therein, incorporated herein by reference)) and ; French Patent Application 2723697) are also encompassed within the invention.

Epstein et al., JACC, 23(6): 1278-88 (1994) and Didier et al. (Rhone Poulenc Rorer SA), French Patent Application, publication no. 2,723,697 (February 23, 1996)

relate to treatments for restenosis, and Chang et al., Science 267:518-522 (January 27, 1995) is directed to therapy for retinoblastoma.

More generally, the antigenic, immunological or vaccine compositions or therapeutic compositions which are prophylactic and/or therapeutic compositions with respect to vascular disease, e.g., atherosclerosis and/or restenosis (compositions containing the vectors or recombinants of the invention or expression products) can be prepared in accordance with standard techniques well known to those skilled in the pharmaceutical or veterinary arts. Such compositions can be administered in dosages and by techniques well known to those skilled in the medical arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient (e.g., factors such as identified in Example 1), and the route of administration. The compositions can be administered alone, or can be co-administered or sequentially administered with other compositions of the invention or with other prophylactic or therapeutic compositions for decreasing viral load or for targeting SMC proliferation.

Such other compositions can include purified native antigens or epitopes or antigens or epitopes from the expression by a poxvirus recombinant or another vector system (such that compositions can contain more that one epitope of interest from CMV and/or p53); antioxidants which inhibit the cytopathic effect of viral infection, and/or compositions which reduce the transcriptional activity of CMV (transcriptional activity reducer) and/or compositions which decrease reactive oxygen species (ROS) generated by the arachidonic cascade and/or the xanthine/xanthine oxidase system (ROS reducer); or another form of molecular based therapy, e.g., expression of cytotoxic molecules to inhibit proliferation of smooth muscle cells and gene therapy, or antisense strategies to inhibit expression of gene products for cell proliferation. Mention is made of WO 96/24604 relating to compositions and

methods for treatment of cardiovascular disease involving genes which are differentially expressed.

The antioxidant can be one or more of Vitamin C, Vitamin E, NAC, PDTC, and the like. For information on ROS, ROS reducers, and antioxidants, mention is made of Ian N. Acworth, Bruce Bailey, "The Handbook of Oxidative Metabolism (ESA, Inc.), e.g., pages i, 1-1, Chapter 2 ("Reactive Oxygen Species"), page 2-1 *et seq.*, Chapter 4 ("Mechanisms of Oxygen Damage"), e.g., page 4-1 *et seq.*, Chapter 5 ("Protection Against Oxidants"), page 5-1 *et seq.*, Chapter 7 ("Diseases Associated With Free Radicals"); Davies, "Oxidative stress: the paradox of aerobic life", Biochem. Soc. Symp. **61**, 1-31; Halliwell, "How to characterize an antioxidant: an update", Biochem. Soc. Symp. **61**, 73-101; all incorporated herein by reference (including documents cited therein).

The transcriptional activity reducer can be an antiviral drug such as gancyclovir and/or acyclovir (which interfere with viral replication), and/or an antioxidant, or the like.

The ROS reducer can be aspirin (acetylsalicylic acid) or a derivative thereof, ASA, indomethacin, oxypurinol, and the like.

Compositions which also can be administered in conjunction with the immunological or vaccine composition in the practice of the invention for prevention or treatment of atherosclerosis and/or restenosis, directed to reducing viral load or burden, include, calcium influx blockers and cyclic nucleotide modulators for inhibiting CMV replication, e.g., as disclosed in U.S. Patents Nos. 4,663,317, 4,800,081, 4,849,412, acyclic pyrrolo[2,3-D pyrimidine analogs, e.g., as disclosed in U.S. Patent No. 4,927,830, polysubstituted benzimidazoles, e.g., as disclosed in U.S. Patent No. 5,360,795, heterocyclic thioamides and analogs, e.g., as disclosed in U.S. Patent No. 5,543,413, or anti-HCMV pharmaceutical compositions, e.g., as disclosed in U.S. Patent No. 5,316,768. Mention

is also made of U.S. Patent No. 5,547,992, relating to anti-HCMV polycarbonate oligomers.

An interesting embodiment can include administration of an antiviral drug such as gancyclovir and/or acyclovir.

Such other composition(s) is (are) administered taking into account the aforementioned factors. It is believed that the present invention provides for the first time the use of compositions which target HCMV and are directed to lowering HCMV viral load or burden, as a means for prevention and/or treatment of vascular disease, e.g., restenosis and/or atherosclerosis. Thus, the aforementioned "other composition(s)" (other than HCMV and/or p53 epitope of interest or recombinant or DNA so expressing vaccine or immunological compositions), in another embodiment of the invention, may be administered for the prevention or treatment of atherosclerosis and/or restenosis, without necessarily also administering a HCMV and/or p53 epitope of interest vaccine or immunological composition.

Examples of compositions of the invention include liquid preparations for orifice, e.g., oral, nasal, anal, genital (e.g., vaginal), vascular and/or SMC, etc., administration such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular, intravenous, intraarterial (e.g., at site of lesion or plaque), intralymphatic, or intraperitoneal administration (e.g., injectable administration) such as sterile suspensions or emulsions. In such compositions the recombinant may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like.

Antigenic, immunological or vaccine compositions, can contain an adjuvant and an amount of the recombinant or expression product or isolated product to elicit the desired response (although embodiments of the invention do not necessarily need to contain an adjuvant; and, in some

instances, embodiments of the invention may be without added adjuvant); or, the gene product or product expressed *in vivo* can be in a form which is exceptionally immunogenic (e.g., a fusion peptide wherein a first portion of the peptide enhances immunogenicity; see, e.g., Huebner et al., WO 96/40718, published December 19, 1996).

In human applications, alum (aluminum phosphate or aluminum hydroxide) is a typical adjuvant. Saponin and its purified component Quil A, Freund's complete adjuvant and other adjuvants are used in research and veterinary applications. Chemically defined preparations such as muramyl dipeptide, monophosphoryl lipid A, phospholipid conjugates such as those described by Goodman-Snitkoff et al., J. Immunol. 147:410-415 (1991) and incorporated by reference herein, encapsulation of the protein within a proteoliposome as described by Miller et al., J. Exp. Med. 176:1739-1744 (1992) and incorporated by reference herein, and encapsulation of the protein in lipid vesicles such as Novasome™ lipid vesicles (Micro Vesicular Systems, Inc., Nashua, NH) can also be used.

The compositions of the invention may be packaged in a single dosage form for immunization by parenteral (i.e., intramuscular, intradermal or subcutaneous) administration or orifice administration, e.g., perlingual (i.e., oral), intragastric, mucosal including intraoral, intraanal, intravaginal, intravenous, intralymphatic, intraarterial (e.g., at site of lesion or plaque), intraperitoneal, and the like administration. And again, the effective dosage and route of administration are determined by the nature of the composition, by the nature of the expression product, by expression level if the vector or recombinant is directly used, and by known factors, such as age, sex, weight, condition and nature of patient, as well as LD_{50} and other screening procedures which are known and do not require undue experimentation.

Dosages of expressed product or isolated product (e.g., isolated from CMV-infected cells) can range from a

few to a few hundred micrograms, e.g., 5 to 500 μg. The inventive vector or recombinant can be administered in any suitable amount to achieve expression at these dosage levels. The inventive vector or recombinant can be administered to a patient or infected or transfected into cells in an amount of about at least $10^{3.5}$ pfu; more preferably about 10^4 pfu to about 10^{10} pfu, e.g., about 10^5 pfu to about 10^9 pfu, for instance about 10^6 pfu to about 10^8 pfu. And, if more than one gene product is expressed by more than one recombinant, each recombinant can be administered in these amounts; or, each recombinant can be administered such that there is, in combination, a sum of recombinants comprising these amounts. Other suitable carriers or diluents can be water or a buffered saline, with or without a preservative. The expression product or isolated product or vector or recombinant may be lyophilized for resuspension at the time of administration or can be in solution.

In plasmid compositions, the dosage should be a sufficient amount of plasmid to elicit a response analogous to the expressed antigen compositions; or expression analogous to dosages in expressed antigen compositions; or expression analogous to expression obtained *in vivo* by recombinant compositions. For instance, suitable quantities of plasmid DNA in plasmid compositions can be 1 ug to 100 mg, preferably 0.1 to 10 mg, but lower levels such as 0.1 to 2 mg or preferably 1-10 ug may be employed. Documents cited herein regarding DNA plasmid vectors may be consulted for the skilled artisan to ascertain other suitable dosages for DNA plasmid vector compositions of the invention, without undue experimentation.

The carrier may also be a polymeric delayed release system. Synthetic polymers are particularly useful in the formulation of a composition having controlled release. An early example of this was the polymerization of methyl methacrylate into spheres having diameters less than one micron to form so-called nano particles, reported

by Kreuter, J., Microcapsules and Nanoparticles in Medicine and Pharmacology, (M. Donbrow, ed.) CRC Press, p. 125-148.

Microencapsulation has been applied to the injection of microencapsulated pharmaceuticals to give a controlled release. A number of factors contribute to the selection of a particular polymer for microencapsulation. The reproducibility of polymer synthesis and the microencapsulation process, the cost of the microencapsulation materials and process, the toxicological profile, the requirements for variable release kinetics and the physicochemical compatibility of the polymer and the antigens are all factors that must be considered. Examples of useful polymers are polycarbonates, polyesters, polyurethanes, polyorthoesters and polyamides, particularly those that are biodegradable.

A frequent choice of a carrier for pharmaceuticals and more recently for antigens is poly (d,1-lactide-co-glycolide) (PLGA). This is a biodegradable polyester that has a long history of medical use in erodible sutures, bone plates and other temporary prostheses where it has not exhibited any toxicity. A wide variety of pharmaceuticals including peptides and antigens have been formulated into PLGA microcapsules. A body of data has accumulated on the adaption of PLGA for the controlled release of antigen, for example, as reviewed by Eldridge, J.H., et al., Current Topics in Microbiology and Immunology, 1989, 146:59-66. The entrapment of antigens in PLGA microspheres of 1 to 10 microns in diameter has been shown to have a remarkable adjuvant effect when administered orally. The PLGA microencapsulation process uses a phase separation of a water-in-oil emulsion. The compound of interest is prepared as an aqueous solution and the PLGA is dissolved in a suitable organic solvents such as methylene chloride and ethyl acetate. These two immiscible solutions are co-emulsified by high-speed stirring. A non-solvent for the polymer is then added, causing precipitation of the polymer around the aqueous

droplets to form embryonic microcapsules. The microcapsules are collected, and stabilized with one of an assortment of agents (polyvinyl alcohol (PVA), gelatin, alginates, polyvinylpyrrolidone (PVP), methyl cellulose) and the solvent removed by either drying *in vacuo* or solvent extraction.

Thus, solid, including solid-containing-liquid, liquid, and gel (including "gel caps") compositions are envisioned.

Furthermore, the vector or recombinant or expression products therefrom or isolated products can be used to stimulate a response in cells *in vitro* or *ex vivo* for subsequent reinfusion into a patient. If the patient is seronegative, the reinfusion is to stimulate an immune response, e.g., an immunological or antigenic response such as active immunization. In a seropositive patient, the reinfusion is to stimulate or boost the immune system against the CMV and/or p53, for prevention or treatment of vascular disease such as restenosis and/or atherosclerosis.

For treatment of restenosis, a HCMV and/or p53 vaccine or immunological composition, alone or with other treatment as herein discussed, may be administered as desired by the skilled medical practitioner, from this disclosure and knowledge in the art, e.g., at the first signs or symptoms of restenosis, or as soon thereafter as desired by the skilled medical practitioner, without any undue experimentation required; and, the administration of the vaccine or immunological composition, alone or with other treatment as herein discussed, may be continued as a regimen, e.g., monthly, bi-monthly, biannually, annually, or in some other regimen, by the skilled medical practitioner for such time as is necessary to boost the immune response against CMV and keep it boosted so as to prevent further clogging of blood vessels or further symptoms or signs of restenosis, without any undue experimentation required.

For prevention of restenosis, a HCMV and/or p53

vaccine or immunological composition, alone or with other treatment as herein discussed, may be administered at the first indication of the patient being prone to restenosis, or as soon thereafter as desired by the skilled medical practitioner, e.g., within six months prior to, immediately prior to, or at angioplasty, such as within six weeks prior to, immediately prior to, or at angioplasty, in any desired regimen such as a single administration or multiple administrations in a regimen as desired, e.g., monthly, bi-monthly, biannually, or any combination thereof, without any undue experimentation required. Further, for prevention of restenosis, a HCMV and/or p53 vaccine composition, alone or with other treatment as herein discussed, may be administered after angioplasty in a regimen of single or multiple administrations as desired by the skilled medical practitioner, such as immediately after, within six weeks after, within six months after, and/or within a year after, e.g., monthly, bi-monthly, biannually, annually, or in some other regimen, by the skilled medical practitioner for such time as is necessary to boost the immune response against CMV and keep it boosted so as to prevent clogging of blood vessels or symptoms or signs of restenosis, without any undue experimentation required.

For treatment of atherosclerosis, a HCMV and/or p53 vaccine or immunological composition, alone or with other treatment as herein discussed, may be administered at the first signs or symptoms of atherosclerosis, or as soon thereafter as desired by the skilled medical practitioner, without any undue experimentation required; and, the administration of the vaccine or immunological composition, alone or with other treatment as herein discussed, may be continued as a regimen, e.g., monthly, bi-monthly, biannually, annually, or in some other regimen, by the skilled medical practitioner for such time as is necessary to boost the immune response against CMV and keep it boosted so as to prevent further clogging of blood vessels

or further symptoms or signs of atherosclerosis, without any undue experimentation required.

For prevention of atherosclerosis, a HCMV and/or p53 vaccine or immunological composition, alone or with other treatment as herein discussed, may be administered at the first indication of the patient being prone to restenosis and/or atherosclerosis, or as soon thereafter as desired by the skilled medical practitioner, in any desired regimen such as a single administration or multiple administrations in a regimen as desired, e.g., monthly, bi-monthly, biannually, or any combination thereof, without any undue experimentation required, e.g., for such time as is necessary to boost the immune response against CMV and keep it boosted so as to prevent clogging of blood vessels or symptoms or signs of atherosclerosis, without any undue experimentation required.

Further, given the prevalence of HCMV in the population as correlated to age, as discussed above (CMV present: in about 10 to 15% of the adolescent population; in about 40 to 50% of the adult, age 35 population; and in more than 60 to 70% of the adult, over age 65 population), a program of administering a HCMV vaccine or immunological composition from childhood, to reduce the prevalence of HCMV in the population, is yet a further method for preventing atherosclerosis and/or restenosis; and, this program can be annual, bi-annual or some other regimen of administration as desired by the skilled medical practitioner, without undue experimentation.

The therapeutic vaccine or immunological composition of the invention can be administered before the angioplasty to induce maximal cellular immune responses at the time of angioplasty, since the restenotic process happens quickly; however, treatment after angioplasty is not excluded.

As discussed above, the present invention also pertains to diagnostic compositions and methods; and, these diagnostic methods and compositions may be used in

conjunction with the therapy and/or treatment and/or prophylactic compositions and methods of the invention.

The method for diagnosis to ascertain a susceptibility to atherosclerosis and/or restenosis can comprise immunologically detecting CMV antibodies, preferably against specific viral proteins that are more specific indicators that the virus has been reactivated, such as IE72, IE84, IE55 and the like. The immunologically detecting can be by ELISA and/or immunoblotting. The Examples below discuss testing patients for antibodies against CMV, as well as testing samples for the presence of CMV epitope(s) of interest, antibodies thereto, and DNA coding therefor. Mention is also made of U.S. Patents Nos. 5,180,813 and 4,716,104, incorporated herein by reference, relating to early envelop glycoprotein and monoclonals to HCMV glycoproteins, and detection of HCMV antigens by antibodies reactive to IE.

The method can include, in addition or alternatively to detecting the neutralizing antibodies, detecting whether CMV mRNA is present in peripheral blood monocytes (PBMCs), e.g., by PCR (such as RT-PCR) and/or detecting whether a cellular-mediated immune response to CMV peptides or proteins is present, e.g., whether PBMCs recognize and/or respond to CMV peptides or proteins.

To detect whether CMV nucleic acids are in a sample, the skilled artisan can employ DNA for primers, as used in the Examples below, or as in the art, e.g., the Paoletti and Paoletti et al. patents and patent publications discussed herein, U.S. Patents Nos. 5,569,583, 5,173,402, and 4,762,780, incorporated herein by reference, relating to detection of CMV using primers or DNA sequences, U.S. Patents Nos. 5,047,320 and 5,075,213, incorporated herein by reference, relating to DNA probes for HCMV gp64 (as well as use of HCMV gp64 as a vaccine), and U.S. Patents Nos. 5,591,439 and 5,552,143, incorporated herein by reference, relating to adenovirus-HCMV gB and IE-exon 4 recombinants.

For instance, DNA as herein disclosed may be contacted with a specimen from a patient, with that DNA employed as a primer in a polymerase chain reaction. From that the skilled artisan can detect the presence or absence of CMV in the sample, and ergo propensity to or against vascular disease such as restenosis and/or atherosclerosis. The sample can be SMCs, sera, blood, or the like, or samples as used in the art.

This aspect of the invention can relate to a skin test whereby the CMV proteins or peptides are administered subcutaneously or intradermally or intramuscularly, which reflects the patient's capacity to mount a cellular-mediated response targeted to the CMV proteins or peptides. A negative or positive skin test shows patients with prior CMV infection and who are thus susceptible or resistant to atherosclerosis and/or restenosis. A negative skin test, for instance, may show either someone who has never seen the virus (Ab-T- of Example 2) or someone who has seen the virus, but did not make a cellular response (Ab+T- of Example 2).

This aspect of the invention can relate more generally to presenting the patient's PBMCs with CMV proteins or peptides and measuring either the proliferative response of the cells or the cytokine profile to determine whether there is a dominant Th1 (e.g., IL-2, IFN-12, IFNγ) or Th2 (IL-4, IL-10) response.

The CMV proteins or peptides can be purified CMV proteins or peptides from lysates of cells previously infected with CMV, or from recombinant expression of the CMV proteins or peptides or epitopes of interest; and, useful in this aspect of the invention is the CMV and p53 epitopes of interest discussed in the following Examples or as in the art, e.g., the Paoletti and Paoletti et al. patents and patent publications discussed herein, U.S. Patents Nos. 5,047,320 and 5,075,213, incorporated herein by reference, relating to HCMV gp64 as a vaccine, and U.S. Patents Nos. 5,591,439 and 5,552,143, incorporated herein

by reference, relating to adenovirus-HCMV gB and IE-exon 4 recombinants and products therefrom.

This aspect of the invention can also relate to HLA phenotyping and/or HLA genotyping, as such phenotyping and/or genotyping can be used to predict the susceptibility to CMV-induced vascular disease such as restenosis and/or atherosclerosis (see, e.g., Example 2).

This aspect of the invention can further relate to detection of p53. CMV interacts with p53 in smooth muscle cells (SMCs). p53 present in increased amounts binds to MHC Class I antigens in the SMCs and is processed and presented at the cell surface at an increased rate, resulting in stimulation of T cell response, underlying the antibody responses (whereas normal p53 is immunologically silent). Increased or steady state levels of p53 are present in cancers or when viral oncoproteins bind to p53 (as is the case with CMV).

Thus, detection of p53, e.g., at lesions, can be indicative of the presence of CMV proteins, and an indicator of the presence or absence or restenosis and/or atherosclerosis, or of the propensity to develop vascular disease such as restenosis and/or atherosclerosis. p53, or an epitope thereon, can be obtained from cells, or by recombinant methods, e.g., as discussed in the Examples, for use in this aspect of the invention; or, for use in this aspect of the invention, one can use antibodies elicited by such p53, or an epitope thereon, for detection of the presence of p53.

Accordingly, the diagnostic method can comprise screening a sample from a patient (e.g., sera, blood, SMCs, lesions) for antibodies to CMV and/or for the presence of CMV proteins and/or p53. The method can further comprise: screening a sample from a patient for specific viral proteins and/or antibodies thereto that predict whether the virus has been reactivated such as IE72, IE84, IE55 and the like.

These screenings can employ epitopes of interest

as in the Examples, or as in the art, e.g., the Paoletti and Paoletti et al. patents and patent publications discussed herein, U.S. Patents Nos. 5,047,320 and 5,075,213, incorporated herein by reference, relating to HCMV gp64, and U.S. Patents Nos. 5,591,439 and 5,552,143, incorporated herein by reference, relating to adenovirus-HCMV gB and IE-exon 4 recombinants, in binding assays, or antibodies elicited therefrom; and, binding assays and purification/isolation procedures with respect to epitopes of interest are included in the Examples, or as in the art, e.g., the Paoletti and Paoletti et al. patents and patent publications discussed herein, U.S. Patents Nos. 5,047,320 and 5,075,213, incorporated herein by reference, relating to HCMV gp64, and U.S. Patents Nos. 5,591,439 and 5,552,143, incorporated herein by reference, relating to adenovirus-HCMV gB and IE-exon 4 recombinants, and U.S. Patents Nos. 5,180,813 and 4,716,104 relating to monoclonals to HCMV glycoproteins and detection of HCMV antigens by antibodies reactive to IE.

These screenings can further comprise detecting whether CMV mRNA is present in PBMCs, e.g., by PCR (such as RT-PCR), e.g., employing DNA as disclosed in the Examples herein, or as in the art, e.g., the Paoletti and Paoletti et al. patents and patent publications discussed herein, U.S. Patents Nos. 5,047,320 and 5,075,213, incorporated herein by reference, relating to HCMV gp64, and U.S. Patents Nos. 5,591,439 and 5,552,143, incorporated herein by reference, relating to adenovirus-HCMV gB and IE-exon 4 recombinants; and/or detecting whether a cellular-mediated immune response to CMV peptides or proteins is present, e.g., whether PBMCs recognize and/or respond to CMV peptides or proteins, e.g., by administering a CMV skin test by administering CMV proteins or peptides intradermally or subcutaneously or intramuscularly and ascertaining the result of the skin test and/or presenting CMV proteins or peptides to a patient's PBMCs and measuring either the proliferative response of the cells (PMBCs) or

the cytokine profile; and/or HLA phenotyping and/or HLA genotyping; and optionally screening a sample from a patient (e.g., sera, blood, lesions, SMCs, etc.) for p53. With respect to RT-PCR (reverse transcriptase-polymerase chain reaction), reference is made to Luehrsen et al., BioTechniques **22(1)**:168-174 (1996).

The initial screening for antibodies to CMV may optionally be omitted, such that the diagnostic method can comprise: screening a sample from a patient for specific viral proteins that predict whether the virus has been reactivated such as IE72, IE84, IE55 and the like; and/or detecting whether CMV mRNA is present in PBMCs, e.g., by PCR (such as RT-PCR); and/or detecting whether a cellular-mediated immune response to CMV peptides or proteins is present, e.g., whether PBMCs recognize and/or respond to CMV peptides or proteins, e.g., by administering a CMV skin test by administering CMV proteins or peptides intradermally or subcutaneously or intramuscularly and ascertaining the result of the skin test and/or presenting CMV proteins or peptides to a patient's PBMCs and measuring either the proliferative response of the cells (PMBCs) or the cytokine profile; and/or HLA phenotyping and/or HLA genotyping; and optionally screening a sample from a patient (e.g., sera, blood, SMCs, lesions, etc.) for p53.

In general, the diagnostic methods are to ascertain the presence of or propensity towards or against vascular disease such as restenosis and/or atherosclerosis which evaluate whether an individual has been infected by CMV and/or whether a cellular response is present, wherein the cellular mediated response may be predictive of an ability to fight infection, e.g., predictive of a predisposition to or against (prevention of) vascular disease such as restenosis and/or atherosclerosis. Alternatively, it may be predictive of immunopathology, and thereby predict susceptibility to restenosis and/or atherosclerosis. The diagnostic methods can be for

stratification of atherosclerosis and/or restenosis risk.

For instance, the methods of the present invention may be useful in the following scenario: someone presents with coronary artery disease and angioplasty is being considered. The patient would be tested for CMV (Abs or cellular response, etc. as herein). If negative, the patient would be at low risk for restenosis (see Examples 1, 2), so angioplasty is indicated without therapy or treatment, e.g., without pre-angioplasty and/or follow-up treatment or therapy, such as aggresive follow-up. If positive, then the patient has a 40-50% risk of restenosis (see Examples 1, 2), and should probably get treatment or therapy, e.g., pre-angioplasty and/or follow-up to angioplasty, by the administration of a composition according to the invention (see description *supra*, Examples 3 *et seq.*), or a combination of both in doses such that the skilled artisan would consider such therapy or treatment "aggressive".

And, the CMV in the various aspects to which the invention pertains can be of human CMV (HCMV), murine CMV (MCMV) or rat CMV (RCMV) origin, with HCMV and RCMV embodiments preferred.

In addition, the therapeutic and prophylactic methods of the present invention can be performed with respect to other infectious agents causing cardiovascular disease. For instance, an antigen or portion thereof, such as an epitope of interest, or a recombinant, e.g., naked DNA, DNA plasmid, virus, etc. expressing such an antigen etc., *in vivo* and/or *in vitro*, of another infectious agent linked to cardiovascular disease may be employed instead of or in addition to the CMV antigen or portion thereof in the present invention.

An example of a particular additional infectious agent is Chlamydia pneumoniae, which has been implicated in coronary artery disease; see, e.g., Peeling et al. Emerging Infectious Diseases 2:307-319 (1996); Saikku et al., Chronic Chlamydia pneumoniae Infection as a Risk Factor for

Coronary Heart Disease in the Helsinki Heart Study. Ann Intern Med 1992;116:273-8; Thom et al., Association of Prior Infection With Chlamydia pneumoniae and Angiographically Demonstrated Coronary Artery Disease. JAMA 1992;268:68-72; Melnick et al., Past Infection by Chlamydia pneumoniae Strain TWAR and Asymptomatic Carotid Atherosclerosis. Am J Med 1993;95:499-504; Shor et al., Detection of Chlamydia pneumoniae in coronary arterial fatty streaks and atheromatous plaques. S Afr Med J 1992;82:158-61; Kuo et al., Demonstration of Chlamydia pneumoniae in Atherosclerotic Lesions of Coronary Arteries. J Infect Dis 1993;167:841-9; Muhlestein et al., Increased incidence of Chlamydia species within the coronoary arteries of patients with symptomatic atherosclerotic versus other forms of cardiovascular disease. J Am Coll Cardiol 1996;27:1555-61; Godzik et al., In Vitro Susceptibility of Human Vascular Wall Cells to Infection with Chlamydia pneumoniae. J Clin Microbiol 1995;33:2411-4 (but see Weiss et al., Failure to detect Chlamydia pneumoniae in coronary atheromas of patients undergoing atherectomy. J Infect Dis 1996;173:957-62, which is discounted in view of the overwhelming foregoing citations to the contrary). Similarly, the diagnostic methods can be extended to detecting the presence of such other infectious agents. And, these additional therapeutic, prophylactic and diagnostic methods are all within the ambit of the present invention.

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

EXAMPLES

EXAMPLE 1 - RELATION BETWEEN ANTIBODIES TO CMV AT ANGIOPLASTY AND RESTENOSIS

With respect to this Example, reference is made to Zhou et al., "Association Between Prior Cytomegalovirus Infection And The Risk Of Restenosis After Coronary Atherctomy," August 29, 1996, New England Journal of

Medicine, 335:624-630, incorporated herein by reference.

CMV infection of immunocompetent adults is common, see Melnick et al. European Heart Journal, *supra*, and usually asymptomatic, Jordan et al., Ann. Intern, Med. 1973; 79:153-160., Klacsmann, De. Med. J. 1977; 49:499-509. Like other herpesviruses, CMV persists indefinitely in certain host cells. Bruggeman, Vurchows Arch. B. Cell Pathol. 1993; 64:325-333; Banks et al., Clin. Infect. Dis. 1992; 14:933-941. Certain circumstances such as immunosuppression, Jacobson et al.. Ann. Intern. Med. 1988; 108:585-94, or iatrogenically following organ transplantation, Schulman et al., Arch. Intern, Med. 1981; 151:1118-24, CMV can be reactivated and cause serious disease, as can other herpesviruses. Viral replication may contribute to the disease process.

CMV may also contribute to disease processes during abortive infections, Southern et al., Engl. J. Med. 1986; 314:359-67, wherein there is viral gene expression limited to immediate early (IE) gene products without viral replication, see Geist et al., Am. J. Respir. Cell. Mol. Biol. 1991; 5:292-296 (CMV IE gene products affecting expression of human cellular genes involved in inflammation and immunologic responses).

Methods

Patients and study design.

Patients included in this investigation were part of the OARS trial (Optimal Atherectomy Restenosis Study), which was designed to determine the frequency of restenosis following directional coronary atherectomy (DCA). Follow-up angiographic evaluation was obtained approximately 6 months later. Patients derived solely from one of the four multicenter sites (Washington Hospital Center), which recruited 100 of the total 211 OARS patients. Of these 100 patients, 7 were "de-registered" due to an initial procedural complication or protocol violation; an additional 18 patients failed to obtain follow-up angiographic study, leaving a total of

75 patients included in this study.

The patients ranged from 35 to 78 years (mean 58), and there were 58 men and 17 women. Blood samples were collected before and six months after DCA to assay anti-CMV IgG and IgM antibody status. Blood samples were assayed for anti-CMV antibodies without knowledge of the patients' angiographic status.

Clinical definitions

The following definitions were used: *diabetes*--if the patient was taking insulin or oral hypoglycemic agents, or had previously taken them and was currently diet controlled; *hypertension*--if the patient was diagnosed as having hypertension and/or was being treated with antihypertensive medications or diet; *hypercholesterolemia*--if the patient had a serum cholesterol value of >240 mg/dl at the time of angioplasty or if the patient was on cholesterol lowering treatment.

Directional atherectomy procedure.

Optimal directional coronary atherectomy involves 1) initial localized plaque resection followed by 2) circumferential plaque resection using larger devices or higher support balloon pressures, and usually concluded with 3) adjunct low-pressure balloon dilatation. Ultrasound guidance is utilized to optimize results. Of the 75 patients, 65 (87%) had adjunct PTCA resulting in a mean 10% additional reduction in final percent diameter stenosis. Two patients (3%) had stents placed after the atherectomy procedure to treat severe lumen-compromising dissections.

Angiographic analysis.

Cineangiograms were forwarded to the core angiographic laboratory blinded to the results of patients' anti-CMV antibody status. Baseline, post DCA procedural, and late follow-up cineangiograms were analyzed using an automated edge detection algorithm (CMS, MEDIS). Minimal lumen diameter (MLD), interpolated

reference diameter, and percent diameter stenosis before and after intervention and on follow-up angiography were measured from two projections; the average of these two values is reported. Acute gain was defined as MLD immediately post DCA minus MLD pre DCA; late loss was defined as MLD immediately post DCA minus MLD at six-month follow-up; loss index was defined as late loss divided by acute gain. Restenosis was defined as a dichotomous endpoint of >50% diameter stenosis at follow-up study in a lesion that had been opened to a <50% narrowing immediately after the DCA procedure.

Assays for CMV antibodies

Anti-CMV IgG assay. Anti-CMV IgG antibodies were tested by using the ELISA kit (Cytomegelisa II test kit) from BioWhittaker (Walkersville, Md) according to manufacturer's directions. Patient antibody titers ("cytomegelisa value") were determined from a standard curve. The threshold value for defining a result as seropositive was determined prospectively, as per the manufacturer: a cytomegelisa value <0.25 units is a negative response, while a value of ≥0.25 units indicates prior exposure to CMV.

Anti-CMV IgM test. Anti-CMV IgM antibodies were tested by using the enzyme-linked antibody capture assay kit (CMV CAP-M) from BioWhittaker (Walersville, MD), according to the manufacturer's directions. As per the manufacturer, an index value of < 0.9 was interpreted as negative for CMV IgM antibodies, while a value of >1.1 was interpreted as positive for CMV IgM.

Statistical analysis

Statistical analyses of frequency counts were performed by the Chi-Square test or the Fisher's Exact test for small sample sizes, and means were compared by the two-sample t-test. All tests were 2-sided. The odds ratio, for comparing the odds of restenosis in those with a given risk factor to those without the risk factor, was chosen as a measure of risk in this prospective study.

Modelling of the dichotomous definition of restenosis outcome was performed using the logistic regression model. Factors affecting loss index were identified using linear regression. The covariates considered were CMV status (as a dichotomous variable), CMV titer (as a continuous variable), diabetes, hypercholesterolemia, hypertension, left anterior descending coronary artery location, small reference vessel size (<3 mm in diameter), a history of recent smoking, gender, age, and whether or not the patient had unstable angina as the indication for DCA. All covariates were examined for importance as predictors of restenosis and loss index univariately, as a group in one multivariate model, and in a stepwise multivariable model.

Patient Characteristics

The patients in this study are of similar age and gender, and have similar vessel lesion distribution as the total OARS cohort (Table 1). suggesting that the subgroup is representative of patients undergoing DCA in the larger study.

Forty-nine of the 75 patients (65%) had positive anti-CMV IgG antibody status at study entry, suggesting that they had prior CMV exposure. This prevalence of CMV seropositivity is similar to that reported in several epidemiologic studies conducted in subjects of similar age. Geist et al., Am. J. Respir. Cell. Mol. Biol. 1991; 5:292-296. Of the 18 patients deleted from study because a 6-month angiogram was not obtained, 11 (61%) were CMV seropositive, a percentage virtually identical to that of the 75 patients included in the study. Restenosis developed in 23 of the 75 patients (31%).

Within the CMV seropositive and seronegative groups the relative prevalence of several factors suspected of conveying some increased risk of developing restenosis (see Table 4) did not differ. The one exception was hypertension, which was present in 59% of

the seropositive and in 31% of the seronegative patients (p=0.02). Additional analyses showed, however, that hypertension was unrelated to restenosis (p=0.18).

Correlation between CMV seropositivity and development of restenosis.

By comparing patients' anti-CMV IgG antibody status at study entry with six month angiographic outcome, we found that of the 49 patients with prior CMV exposure, 21 (43%) developed restenosis; only 2 of the 26 patients (8%) without prior CMV exposure developed restenosis (p=0.002; Figure 1A). Analysis of the data using percent stenosis of target vessels at follow-up as a continuous variable indicated that CMV infection predisposes to more severe stenosis (p=0.01; Figure 1, Table 2).

The luminal dimensions and percent stenosis at baseline, immediately after the DCA procedure, and at follow-up are presented in Table 2. A plot of the cumulative percent of target vessels against MLD at each of the three time points, is shown in Figure 2. At baseline, the reference vessel diameter and lesion MLD tended to be larger in the CMV seropositive patients, but there was no difference in percent stenosis. Immediately after the procedure, the seropositive group had a slightly larger lesion MLD (p=0.01), but the mean acute gain was similar. However, the seropositive group had a much greater late loss (p=0.003) and, most importantly, an almost 50% greater loss index than the seronegative group (p=0.0005; Table 2 and figure 3).

The influence of CMV seropositivity and other risk factors on the development of restenosis.

Univariate analyses (Table 3) identified CMV status as the only statistically significant predictor of restenosis (odds ratio=9.0, p=0.002). An analysis of the association of mean IgG antibody titers on restenosis confirmed the finding (mean titer = 0.66 ± .30 units for restenosis patients and 0.44 ± .35 for no restenosis;

p=0.01). There were no other statistically significant predictors of restenosis among the remaining potential risk factors examined. CMV status and CMV titer maintained their relationship with restenosis in the full multivariate logistic regression models (odds ratios, with 95% confidence intervals: =12.9; 2.3, 71.11, p=0.003, and =8.1; 1.5, 43.2, p=0.01, respectively).

The influence of CMV seropositivity and other risk factors on loss index.

Simple linear regression models show that both CMV titer and the dichotomous CMV status (cytomegelisa values ≥0.25 considered positive for CMV, as defined prospectively) are each strong predictors of loss index (p=0.01 and p=0.002, respectively).

The full multiple regression model for loss index shows CMV, when analyzed either as a continuous titer or a dichotomous variable, to be a persistent and independent predictor over and above the effects of all other model covariates (p=0.03 and p=0.01, respectively). Table 4 contains the results for the full model with CMV titer. No other risk factors gained or lost appreciable importance between univariate and multivariate analysis. Also, a stepwise approach to model selection identified CMV titer (and CMV status) as the only significant prognostic variable for loss index. Although the relationship between CMV titer and restenosis was highly significant (p=0.01), CMV titer explained only 7% of the variation in late loss index (r^2=0.07). To put this into perspective, taken as a whole, *all* the risk factors analyzed in this investigation explain only 11.5% if the total variation in loss index.

To determine whether the effect of CMV differed in subgroups defined by the other potential risk variables analyzed in the study, a two-factor interaction of each with CMV was tested and none found significant.

Evidence against the presence of acute infection and systemic viremia.

Assays for anti-CMV IgM antibodies, usually present only early after acute infection, were performed. No anti-CMV IgM antibodies were detected in any of the patients. Also, at approximately the six month time-point of the study (the time of follow-up angioplasty), a second assay to determine IgG anti-CMV antibody titers was performed. There was no significant change in titers (Figure 4). Most importantly, no patient in the original CMV immunopositive group exhibited a significant increase in titer (>2x), and titers fell to within the negative range in only four CMV seropositive patients (of these four patients, all developed restenosis). In addition, none of the original CMV seronegative patients became seropositive.

Immune status against another virus.

To determine whether the correlation between CMV immunopositivity and restenosis was merely a reflection of either a generalized susceptibility to viral infection or a marker of an increased but non-specific immune responsiveness, we determined whether there was a correlation between pre-existing antibodies to Hepatitis A virus and restenosis (seroposivity to Hepatitis A has approximately the same frequency as seropositivity to CMV). Forty-one percent of the total patient group was seropositive for Hepatitis A virus. However, no significant association with restenosis was found; the restenosis rate was 35.7% for Hepatitis A seropositive patients and 37.5% for Hepatitis A seronegative patients.

This Example provides the first prospective evidence indicating that prior exposure to CMV, as indicated by the presence of CMV IgG antibodies, at the time of coronary angioplasty, is a strong independent risk factor for the subsequent development of restenosis ($p = 0.002$; Figure 1).

Table 1. Comparison between total patient cohort of OARS and the OARS subgroup included in the present study.

	Total OARS (N = 199)	Subgroup studied (N = 75)	P value
Age	58 ± 11 (36-80)	58 ± 10 (35-78)	100♦
Gender (male)	152 (76%)	58 (77%)	0.868†
SVD+DVD*	187 (94%)	73 (97%)	0.525+

*SVD, DVD = number of patients with single and double vessel disease respectively.

♦By 2-sample T-test (two tailed)

†By X^2-text

+By Fisher's Exact test (two tailed)

Table 2. Influence of anti-CMV IgG seropositive/seronegative status on angiographic results of atherectomy

	CMV + (N = 58 vessels) Mean ± SD mm	CMV - (N = 27 vessels) Mean ± SD mm	P-value*
PRE			
Reference diameter	3.23 ± 0.42	3.05 ± 0.48	0.07
MLD	1.29 ± 0.44	1.09 ± 0.33	0.045
Stenosis (%)	60 ± 12	64 ± 11	0.21
IMMED-POST			
Reference diameter	3.37 ± 0.44	3.21 ± 0.47	0.13
MLD	3.18 ± 0.51	2.89 ± 0.45	0.01
Stenosis (%)	5 ± 13	10 ± 10	0.11
FOLLOW-UP			
Reference diameter	3.27 ± 0.49	3.08 ± 0.40	0.08
MLD	1.93 ± 0.94	2.20 ± 0.6	0.12
Stenosis (%)	42 ± 25	28 ± 18	0.01
GAIN/LOSS			
Acute gain	1.90 ± 0.56	1.80 ± 0.55	0.44
Late loss	1.24 ± 0.83	0.68 ± 0.69	0.003
Loss index (%)	68 ± 47	36 ± 33	0.0005

* by 2-sample T-test (two sided)

Reference diameter refers to diameter of the normal segment of vessel adjacent to the stenosis.

MLD = minimal luminal diameter of the stenotic lesion

Definition of gain/loss terms as per Example

Table 3. Univariate association of restenosis with potential risk factors.

	Restenosis* (n = 23)	No restenosis (n = 52)	Odds Ratio (95% CI)	P Value*
	N (%)	N (%)		
DMV +	21 (91%)	28 (54%)	9.00 (1.91, 42.38)	0.002
Diabetes	4 (17%)	8 (15%)	1.16 (0.31, 4.31)	1.00'
LAD lesion	11 (48%)	25 (48)	0.99 (0.37, 2.64)	0.98
Vessel size (<3 mm dia)	8 (35%)	21 (40%)	0.79 (0.28, 2.19)	0.65
Hypertension	14 (61%)	23 (44%)	1.96 (0.72, 5.33)	0.18
Hypercholesterolemia	7 (30%)	21 (40%)	0.65 (0.23, 1.84)	0.41
Smoking	5 (22%)	17 (33%)	0.57 (0.18, 1.8)	0.34
Gender (men)	20 (87%)	38 (73%)	2.44 (0.63, 9.09)	0.19
Unstable angina	17 (74%)	40 (77%)	0.85 (0.27, 2.64)	0.78

* Restenosis defined as dichotomous variable (>50% luminal diameter narrowing)

+ All p-values by x^2-text except' by Fisher's Exact Test (two-tailed).

Table 4. Association of potential risk factors with loss index (Full multiple linear regression model)

Risk factor	Slope	p value
CMV titer*	0.36	0.025
Diabetes	-0.03	0.83
LAD lesion	0.09	0.42
Vessel size (<3 mm dia)	-0.03	0.78
Hypertension	-0.06	0.62
Hypercholesterolemia	0.06	0.58
Unstable angina	-0.06	0.64
Smoking	-0.03	0.81
Gender (male)	-0.13	0.35
Age	0.01	0.30

*When CMV status is defined as a dichotomous value the association when loss index is even stronger ($p = 0.007$) than when defined as titer, a continuous variable.

EXAMPLE 2 - IMMUNODOMINANT CELLULAR AND HUMORAL RESPONSES TO CMV AND THEIR REGULATION BY SPECIFIC HLA ALLELES

Human cytomegalovirus (CMV) rarely produces clinically recognizable disease in immunocompetent individuals. However, like other herpesviruses, it persists in the infected host for life and, under certain circumstances, can be reactivated to cause clinically important disease. Most known CMV-related diseases occur in immune-compromised patients--such as the CMV-associated diseases experienced by many patients following organ transplantation (R. H. Rubin and R. B. Colvin, in *Kidney transplant rejection; Diagnosis and treatment*, G. M. Williams, J. F. Burdick, K. Solez Eds. (New York: Dekker, 1986) pp. 283), and the CMV-induced diseases that complicate the course of AIDS patients (R. D. Schrier, W. R. Freeman, C. A. Wiley, J. A. McCutchan, and the HNRC group, J. Clin. Invest. **95**, 1741 (1995)).

Clinically important CMV-induced disease, however, may not be limited to immune-compromised subjects, as Example 1 provides the first prospective evidence indicating that prior exposure to CMV, as indicated by the presence of CMV IgG antibodies, at the time of coronary angioplasty, is a strong independent risk factor for the subsequent development of restenosis ($p = 0.002$; Figure 1); with respect to CMV and the development of vascular diseases such as restenosis following coronary angioplasty, and atherosclerosis, see E. Speir et al., Science **256**, 391 (1994); Y. F. Zhou et al., N. Engl. J. Med. **335**, 624 (1996); J. L. Melinick, B. L. Petrie, G. R. Dreesman, J. Burek, C. H. McCollum, M. E. DeBakey, Lancet **2**, 644 (1983); M. T. Grattan, C. E. Moreno-Cabral, V. A. Starnes, P. E. Oyer, E. B. Stinson, N. E. Shumway, JAMA. **261**, 3561 (1989); L. Melnick, E. Adam, M. E. DeBakey, JAMA. **263**, 2204 (1990).

With CMV related to these diseases, it is of interest that many more individuals exhibit evidence of prior CMV infection than develop vascular disease. Applicants therefore speculated that certain hosts infected with CMV, although immunocompetent, lack an efficient immune-surveillance system targeted to CMV, and, thereby, have an impaired capacity to eliminate the virus or to prevent its reactivation from latency.

To test this prediction, Applicants determined whether, in immunocompetent individuals, there is a spectrum of humoral vs cellular immunodominant responses to CMV infection. In addition, evidence in studies of patients with HIV and patients with malaria indicate there is a relationship between human leucocyte antigen (HLA) phenotypes to both the type of immunodominant response and the susceptibility or resistance to disease (S. Rowland-Jones et al., Nat. Med. **1**, 59 (1995); R. D. Schrier, W. R. Freeman, C. A. Wiley, J. A. McCutchan, and the HNRC group, J. Clin. Invest. **95**, 1741 (1995); A. S. Hill et al., Phil. Trans. R. Soc. Lond. B. **346**, 379

(1994); A. S. Hill et al., Nature **360**, 434 (1992)).

Applicants therefore also determined whether, if divergent immune responses to CMV were found in the study population, the type of response is related to HLA phenotypes. Based on data indicating an association between specific HLA phenotypes and 1) cellular immune protection against the development of AIDS in HIV exposed subjects (S. Rowland-Jones et al., Nat. Med. **1**, 59 (1995)), 2) susceptibility to CMV-induced retinitis in patients suffering from AIDS (R. D. Schrier, W. R. Freeman, C. A. Wiley, J. A. McCutchan, and the HNRC group, J. Clin. Invest. **95**, 1741 (1995)), and 3) susceptibility to CMV-induced disease in renal transplant patients (G. Blancho, R. Josien, D. Douiliard, J.D. Bignon, A. Cesbron, J.P. Soulillou, Transplantation **54**, 871 (1992); Y.J. Kraat, M.H.L. Christiaans, F.H.M. Nieman, P.M. van den Berg-Loonen, J.P. van Hooff, C.A. Bruggeman, Lancet **341**, 494 (1993).14, 15), Applicants prospectively examined the hypothesis that in immunocompetent individuals with prior CMV exposure the presence of a cellular immune response to CMV would be associated with HLA-B35, whereas its lack would be associated with HLA-DR7 and HLA-B44.

Fifty healthy individuals who volunteered, under an NIH IRB-approved protocol, to donate blood to the Transfusion Medicine Department, National Institute of Health (NIH) were entered into this study. They consisted of 32 (64%) men and 18 (36%) women, and 32 (64%) Caucasians, 17 (34%) Blacks and 1 (2%) Asian. Their ages ranged from 25 to 62 years (mean 40). The HLA frequencies in these study individuals were similar to the reported HLA frequencies in the North American population (T. D. Lee, in *The HLA system; Distribution of HLA antigens*, J. Lee, Ed. (New York: Springer-Verlag, 1990), pp. 141) (see also below).

To determine whether there are immunodominant humoral and cellular responses to CMV antigens in healthy

individuals, all blood samples were tested for 1) anti-CMV IgG antibodies, using an enzyme-linked immunosorbent assay (ELISA), and 2) the ability of T lymphocytes, obtained from peripheral blood mononuclear cells (PBMCs), to proliferate in response to CMV antigens.

In particular, a blood sample from each individual was obtained from the Transfusion Medicine Department, NIH (Bethesda, MD). PBMCs were separated from whole blood on lymphocyte separation medium (Organon Teknika Corp., Durham, NC) by centrifugation at 1,800 rpm for 25 min at room temperature. The separated cells were collected and washed twice in PBS (Gibco, Laboratories, Grand Island, NY). The number of viable cells was determined by trypan blue exclusion and hemacytometer. PBMCs were then cryopreserved in aliquots in liquid nitrogen until used.

CMV antigens were derived from CMV-infected human fibroblasts.

In particular, Human CMV, Towne strain, was obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and grown in human fibroblasts (HEL299; ATCC) for preparation of the viral antigens. Growth media consisted of Minimum Essential Medium (Gibco) supplemented with 2% fetal bovine serum and antibiotics. Virus titer was measured on HEL299 cells.

The published protocols for CMV antigen preparations were followed, and were as follows:

Briefly, CMV antigens were prepared with 1) heat inactivated CMV (1 hour at 56°C) that was obtained from supernatants of CMV-infected fibroblasts--final concentration of virus was 10^5 plaque-forming units (pfu) before inactivation (R. D. Schrier et al., in Y. F. Zhou et al., N. Engl. J. Med. **335**, 624 (1996)); 2) cell lysates of CMV-infected fibroblasts by repeated freezing and thawing (G.J. Boland, R.J. Hene, C. Ververs, M.A.M. De Haan, G.C. De Gast, Clin. Exp. Immunol. **94**, 306 (1993); and 3) 0.08% glutaraldehyde fixed CMV-infected

fibroblast cells (P.J. Converse, A.D. Hess, P.J. Tutschka, G.W. Santos, Infect. Immun. **41**, 1226 (1983). Both cell lysates and fixed cells were prepared from 2 x 10^6/ml cells by infecting a 90% confluent monolayer of human fibroblasts with CMV at a multiplicity of infection (MOI) of 10. Cells were collected by centrifugation when they showed 50% cytopathic effect. The large stocks were aliquoted and stored at -70°C. Controls for the CMV antigens were obtained from noninfected fibroblasts (mock-infected cells), prepared exactly as described for CMV-infected cells.

Anti-CMV IgG antibodies were detected in 23/50 (46%) of individuals, and CMV-induced T lymphocyte proliferative responses developed in 21/50 (42%). No proliferative response was observed in these individuals when their PBMCs were stimulated with antigens derived from mock-infected fibroblasts, or cultured with medium alone.

Positive controls included: 1) 3 days of stimulation with PHA (Gibco) diluted 1:200; 2) influenza A/Bangkok RX73 (grown in embryonated eggs and used as infectious allantoic fluid at an infectivity of $2x10^4$ tissue culture infectious $dose_{50}$/well) at a final dilution of 1:1,000; 3) Candida antigen (Greer Laboratories, Inc., Lenoir, NC), at a final dilution of 20 mg/ml; 4) a pool of irradiated (5,000 rad) PBMCs from three unrelated healthy donors (2 x 10^6/ml). Negative controls were derived from non-infected (mock-infected) fibroblasts and culture medium alone.

The positive proliferative responses to other antigenic stimuli were: 29/50 (58%) to influenza A plus candida antigens, and 35/50 (70%) to allogenic cells. All 50 individuals responded to phytohaemagglutinin (PHA).

Figure 5 shows the patterns of anti-CMV IgG antibodies and T lymphocyte proliferation to CMV antigens. Of the 50 individuals, nine (18%) had both

anti-CMV IgG antibodies and a T-cell proliferative response to CMV antigens (referred to as the antibody positive/T lymphocyte proliferation positive subgroup). Fourteen (28%) who had anti-CMV antibodies did not show a CMV-induced T-lymphocyte response (referred to as the antibody positive/T lymphocyte proliferation negative subgroup). There were 15 individuals (30%) who were negative for both antibodies and T lymphocyte proliferation to CMV (referred to as the antibody negative/T lymphocyte proliferation negative subgroup).

Unexpectedly, 12 (24%) individuals who did not produce anti-CMV IgG antibodies had positive proliferative responses to CMV antigens (referred to as the antibody negative/T lymphocyte proliferation positive subgroup).

These results demonstrate that immunodominant phenotypes directed against CMV are present in immunocompetent individuals. Of interest, 44% of the 27 individuals who were seronegative for CMV antibodies (and therefore, by conventional criteria, would not be considered to have been exposed to CMV) had T lymphocyte proliferative responses to CMV antigens. This particular subgroup, which displayed a dominant cellular immune response to CMV, constituted 24% of the total population.

To determine whether the immune response to CMV infection is related to specific HLA phenotypes, allelic frequencies for HLA class I and class II molecules were analyzed.

The frequency in the North American population (T. D. Lee, in *The HLA system; Distribution of HLA antigens*, J. Lee, Ed. (New York: Springer-Verlag, 1990), pp. 141) of the specific HLA alleles we prospectively examined is 24% for B44, 26% for DR7, and 18% for B35. There were no significant differences in the HLA allelic frequencies between this control population and the total population, which had allelic frequencies of 30% for B44, 28% for DR7, and 14% for B35. Nor were there significant

differences in allelic frequencies between either of these two groups and the antibody negative/T lymphocyte proliferation negative subgroup, which had allelic frequencies of 40% for HLA-B44, 27% for DR7 and 7% for B35 (Fig. 6D). This latter subgroup can probably be considered to consist of individuals who have not been exposed to CMV infection (although some may have had a prior infection following which the virus was either successfully cleared or has remained latent).

In contrast, the remaining subgroups, characterized by their immunodominant response to CMV antigens, demonstrated marked differences in HLA allelic frequency when compared to that of the North American population or the total study population. Thus, neither of the two antibody-positive groups (one characterized by a positive T lymphocyte proliferative response to CMV antigens (Fig. 6A) and the other with a negative proliferative response (Fig. 6B)) contained any individuals carrying the HLA-B35 allele ($P<0.05$ vs North American and total study populations).

Conversely, in the cellular immunodominant subgroup (CMV-seronegative individuals who were positive for CMV-induced T lymphocyte proliferation; Fig. 6C), none carried HLA-B44, only 8% had DR7, but 50% carried HLA-B35. Both the lower frequency of HLA-B44 (but not DR7) and the higher frequency of HLA-B35 in this cellular immunodominant subgroup are significantly different from the corresponding allelic frequencies in our total study population ($P=0.03$ for HLA-B44 and $P=0.01$ for B35) and in antibody negative/proliferation negative individuals ($P=0.02$ for HLA-B44 and $P=0.02$ for B35). Although the difference remained highly significant when the allelic frequency for HLA-B35 was compared to that of the North American population, that for HLA-B44 was only of marginal significance ($P=0.01$ for B35 and $P=0.08$ for HLA-B44).

To determine whether carrying the HLA-B35

allele uniquely predisposes to a cellular immune response to CMV, the relative frequency of a positive T-cell proliferative response to CMV antigens of those individuals with and those without HLA-B35 was compared. A total of 7 individuals carried HLA-B35, and all were CMV-seronegative. Most importantly, 6 of these 7 (86%) had positive T lymphocyte proliferative responses to CMV antigens (Fig. 7). This is in contrast to 6/20 (30%) of the seronegative individuals without B35 (P=0.02).

Applicants also determined the presence of additional HLA alleles (18 HLA-A alleles, 25 HLA-B, 8 HLA-Cw, 11 HLA-DR, 7 HLA-DRw and 8 HLA-DQ) not prospectively identified as potential determinants of immunodominant response.

Additional HLA phenotypes analyzed were: A1-3, A11, A23, A24, A26, A28-34, A36, A66, A68, A74; B7, B8, B13, B14, B18, B27, B37-42, B51, B53, B55, B57, B58, B60-63, B70-72, B81; Cw1-8; DR1, DR3, DR4, DR9-15, DR18, DRw52, DRw53, DRw3*01-3*03, DRw4*01, DRw5*01 and DQ1-8.

Analysis failed to reveal any significant correlations with cell or antibody immunodominant responses.

Without wishing to necessarily be bound by any one particular theory, Applicants do not rule out that the association between HLA-B35 and a cellular immunodominant response to CMV may be due to a closely-linked but unrelated gene. However, it is of note that HLA-B35, which now has been identified as consisting of a large family of homologous gene products, also is associated with an immunodominant cellular response characterized by the presence of cytotoxic T lymphocytes (CTLs) in subjects exposed to HIV-1 or HIV-2 (S. Rowland-Jones et al., Nat. Med. **1**, 59 (1995)), and with the recognition of epitopes of the *Plasmodium falciparum* malaria parasite, resulting in the generation of specific CTLs (A. S. Hill et al., Phil. Trans. R. Soc. Lond. B. **346**, 379 (1994)). The data from these studies further

suggested that the cellular immune responses associated with HLA-B35 conveyed protection against the development of AIDS (Rowland et al., *supra*) and of severe malaria (Hill et al., *supra*).

Applicants findings demonstrate the association of HLA-B35 with T cell proliferative responses to CMV antigens. This proliferative response has not been shown to be restricted by $CD4^+$ and/or $CD8^+$ T cells, it is noteworthy that the most common CMV-specific CTLs present in CMV-seropositive healthy blood donors was recently demonstrated to be targeted to pp65, a CMV matrix protein, which was found to contain at least three pp65-specific CTL peptides restricted by HLA-B35. CTLs of seronegative individuals may target the same or different CMV proteins.

It has been pointed out that the high polymorphism and redundancy of the mammalian MHC makes it difficult to identify a particular MHC haplotype determining resistance or susceptibility to an infectious pathogen in humans. Although Applicants have not demonstrated a correlation between HLA phenotype and resistance or susceptibility to CMV-related disease, these results demonstrate that some immune competent individuals are genetically predisposed, in an HLA dependent manner, to respond to CMV with a cellular immune response in the absence of a humoral response. Given that the same HLA molecule that predisposes to a cellular immunodominant response to CMV is also associated with a cellular immune response targeted to HIV and to the *P. falciparium* parasite (which seems to convey a protective effect in these diseases), these results have much broader implications.

Specific HLA molecules, such as HLA-B35, may have unique attributes that facilitate the development of a cellular immunodominant response, implying a mechanism whereby some individuals are resistant to certain infectious diseases (or to cancer), and some are

susceptible to the development of diseases characterized by immunopathology (chronic granulomatous diseases and autoimmune disease).

There may be a correlation between this pattern of immune response and either protection from, or exacerbation of, any disease processes caused by CMV. Thus, novel therapeutic strategies, such as herein arise. For instance, these results allow for favorably altering disease outcome by directing attempts to change the immunodominant phenotype from one that increases disease susceptibility to one that promotes resistance.

More importantly, this Example shows that diagnosis for a predisposition towards restenosis from angioplasty or for a predisposition towards atherosclerosis cannot be predicated on merely whether an individual has antibodies against CMV, i.e., any prior correlations between CMV and vascular disease fail to teach or suggest the methods and compositions for diagnosis and therapy or treatment or prophylaxis of the present invention. For instance, this Example demonstrates that detecting cellular immune responses and/or HLA genotyping and/or phenotyping can provide surprisingly better diagnosis. Detection of a cellular mediated response can be more predictive or predisposition to or against (prevention) of restenosis and/or atherosclerosis, since antibody-negative patients, as herein demonstrated can have T-cell responses.

Further, this Example, with Example 1 shows the importance in therapy or treatment or prophylaxis to boost the immune response to CMV and/or p53. Simply, the latent CMV infection is a low grade viral infection that the body cannot rid itself of because there is not sufficient stimulation of immune responses. Therapy, treatment or prophylaxis with a vaccine or immunological composition against CMV and/or p53 can thus boost the immune response to knock out low levels of CMV from the body, and thus provide therapy, treatment or prophylaxis

with respect to restenosis and/or atherosclerosis.

EXAMPLE 3 - POXVIRUS-CMV RECOMBINANTS

Reference is made to PCT WO 96/39491, incorporated herein by reference, with respect to this Example, especially the Examples thereof from Example 12, and the Figures thereof cited in those Examples such as Figures from Figure 12, and Figure 8 thereof.

EXAMPLE 4 - POXVIRUS-p53 EPITOPE OF INTEREST RECOMBINANTS

Reference is made to WO 94/16716, incorporated herein by reference, with respect to this Example, especially Examples 15, 32 and 33, and Figures 17, 18, 38 and 39 of WO 94/16716.

EXAMPLE 5 - POXVIRUS-RAT CMV IE1 AND IE2 RECOMBINANTS

Plasmids RCMVIE1 and RCMVIE2 were obtained from Dr. Toren Finkel (NIH-NHLBI), and transformed into bacteria MN522 (available from Stratgene). In Figure 8 RCMVIE1 the coding sequence for the Rat CMV IE1 gene is depicted from nucleotides 443-2140 (SEQ ID NO:47). In Figure 9 RCMVIE2 the coding sequence for the Rat CMV IE1 gene is depicted from nucleotides 443-2002 (SEQ ID NO:48).

Oligonucleotides SPIE1C (5'-TAG-ATA-AAG-CTG-CAG-AGT-CA-3') (SEQ ID NO:176) and SPIE1D (5'-AGA-CTC-GAG-ATA-AAA-ATT-ATG-ATC-TCC-TGC-CTC-TCT-3') (SEQ ID NO:177) were used in PCR with plasmid RCMVIE1 to generate a 585bp fragment containing the C-terminal end of the IE1 gene. This fragment was digested with PstI and XhoI (yielding a 565bp fragment) and cloned into BamHI/XhoI digested and alkaline phosphatase treated IBI25 along with a 1132bp BamHI/PstI fragment from RCMVIE1 generating plasmid IE1-2-21.

Oligonucleotides SPIE2C (5'-CGC-AAG-CTT-CGC-GAT-AAA-AAT-TAT-TCT-GAA-TCG-GAG-TCC-T-3') (SEQ ID NO:178) and SPIE2D (5'-ATG-ATA-ATC-CAA-GCG-GCA-ACA-3') (SEQ ID NO:179) were used in PCR with plasmid RCMVIE2 to generate a 272bp fragment containing the C-terminal end of the IE2

gene. This fragment was digested with NsiI and PstI (yielding a 210bp fragment) and cloned into BamHI/HindIII digested IBI25 along with a 1361bp BamHI/NsiII fragment from RCMVIE2 generating plasmid IE2-2-4.

Plasmid IBI25 was digested with EcoRI and XbaI, treated with alkaline phosphatase and ligated to kinased and annealed oligonucleotides SPIE2A (5'-CTA-GAG-GAT-CCA-TTT-TAT-ATT-GTA-ATT-ATA-TAT-TTT-CAA-TTT-TGA-AAT-CCC-AAA-ACC-CGG-GAG-ATC-TG-3') (SEQ ID NO:180) and SPIE2B (5'-AAT-TCA-GAT-CTC-CCG-GGT-TTT-GGG-ATT-TCA-AAA-TTG-AAA-ATA-TAT-AAT-TAC-AAT-ATA-AAA-TGG-ATC-CT-3') (SEQ ID NO:181) yielding plasmid IE2-1-1.

Plasmid IE2-1-1 was digested with BamHI and HindIII, treated with alkaline phosphatase and ligated to a 1570bp BamHI/HindIII fragment derived from plasmid IE2-2-4 yielding plasmid IE2-3-1 which contains the Rat CMV IE2 gene under the control of the entemopoxvirus 42K early promoter.

NYVAC donor plasmid pSD553 (which contains the K1L host range gene, a polylinker and sequences flanking the ATI locus; see U.S. Patent No. 5,494,807) was digested with BamHI and NruI, treated with alkaline phosphatase and ligated to a 1618bp BglII/NruI fragment from plasmid IE2-3-1 generating plasmid IE2-4-16.

Plasmid MCP1-3 (which contains the vaccinia early/late H6 promoter) was derived from SPHA-H6. Plasmid SPHA-H6 was used in PCR with oligonucleotides SPMCP1 (5'-GCCTCTAGACTCGAGCGCCGACCAGTTCTCCATTACGATACAAACTTAACGGATATC-3') (SEQ ID NO:184) and SPMCP2(5'-CGCGAATTCTTCTTTATTCTATACTTA-3') (SEQ ID NO:185) and the resulting 166 bp fragment was digested with Eco RI and XbaI and ligated to EcoRI/XbaI digested and alkaline phosphatase-treated IBI24 generating plasmid MCP1-3.

Plasmid MCP1-3 was digested with EcoRV (within the H6 promoter) and XbaI (within the polylinker), treated with alkaline phosphatase and ligated to kinased

and annealed oligonucleotides SPIE1A (5'-ATC-CGT-TAA-GTT-TGT-ATC-GTA -ATG-GAT-CCT-3') (SEQ ID NO:182) and SPIE1B (5'-CTA-GAG-GAT-CCA-TTA-CGA-TAC-AAA-CTT-AAC-GGA-T-3') (SEQ ID NO:183) yielding plasmid IE1-1-3.

Plasmid IE1-1-3 was digested with BamHI and XhoI, treated with alkaline phosphatase and ligated to a 1703bp BamHI/XhoI fragment from plasmid IE1-2-21 yielding plasmid IE1-3-2 (which contains the Rat CMV IE1 gene under the control of the vaccinia H6 promoter).

Plasmid IE2-4-16 was digested with SmaI and XhoI and treated with alkaline phosphatase. Plasmid IE1-3-2 was digested with EcoRI, filled in with Klenow, digested with XhoI and a 1838bp fragment isolated. Ligation of these two fragments yielded plasmid COPAKIE1.2-2. The DNA sequence of Rat CMV IE1 and IE2 plus additional flanking DNA sequences in plasmid COPAKIE1.2-2 is shown in Figures 10A and B providing the nucleotide sequence (DNA) of COPIE1_2 (SEQ ID NO:49). The H6 promoted Rat CMV IE1 gene is located between nucleotides 2252 and 431. The 42K promoted Rat CMV IE2 gene is located between nucleotides 2261 and 3862.

Plasmid COPAKIE1.2-2 was transfected into NYVAC infected CEF cells to generate recombinant vP1479. Analysis confirms expression.

EXAMPLE 6 - BACULOVIRUS RAT CMV IE1 OR IE2 RECOMBINANTS

Baculovirus recombinants expressing Rat CMV IE1 or IE2 were derived using the BAC-TO-BAC BACULOVIRUS EXPRESSION SYSTEM (Life technologies) as described in the instruction manual. This system is based on the site specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli*. The recombinant bacmid DNA is isolated and used to transfect insect cells. Viral stocks harvested from transfected cells are amplified and used to infect insect cells for subsequent protein expression, purification (by virtue of the His tag present on the recombinant protein)

and analysis (see Figure 11, Generation of recombinant baculovirus and gene expression with the Bac-to-Bac Expression system).

The donor plasmid pFASTBACHTa (Figure 12) was digested within the multiple cloning sites (Figure 13) with BamHI and HindIII and a 4771bp fragment isolated. Plasmid IE1-2-21 was digested with BamHI and HindIII and a 1716bp fragment isolated. Ligation of these two fragments yielded plasmid BacRIE1-3 which encodes a fusion protein containing 25 amino acids derived from pFASTBACHTa and the entire rat CMV IE1 amino acid sequence.

Plasmid IE2-2-4 was digested with BamHI and HindIII and a 1570bp fragment was isolated and ligated to the 4771bp BamHI/HindIII fragment from pFASTBACHTa yielding plasmid BacRIE2-4. This plasmid encodes a fusion protein containing 25 amino acids derived from pFASTBACHTa and the entire rat CMV IE2 amino acid sequence.

BacRIE1-3 and BacRIE2-4 were transformed into DH10Bac cells and transposition allowed to occur. Recombinant bacmid DNA was isolated from appropriate colonies and used to transfect Sf9 insect cells to generate recombinant baculoviruses A6 (Rat CMV IE1 recombinant) and B2 (Rat CMV IE2 recombinant). Analysis confirms expression (Figure 15A, lane 6).

Figure 14 (SEQ ID NO: 50) provides the nucleotide sequence (DNA) of HCMV IE2, which is useful in generating vectors or recombinants for use in this invention.

Proteins expressed by the recombinant baculovirus were isolated and purified as follows:

Purification of Recombinant Proteins Expressed by Baculovirus

Baculovirus proteins were purified using the His Trap chelating column from Pharmacia Biotech. A suspension culture of SF9 insect cells at a density of

$2X10^6$ per ml was inoculated with recombinant baculovirus at a multiplicity of 1 plaque forming unit of virus per cell. Cells were incubated at 28° and harvested at 72 hours post infection. Cells were spun out at 2000 rpm for 10 minutes at 4°C and stored at -80°C until processing. Cells were lysed using 5ml of lysis buffer per gram of cells. Lysis buffer was composed of 1X Phosphate buffer (supplied with kit), 10mM Imidazole (supplied with kit), 1% NP-40, 1mM PMSF, and 0.01M Mercaptoethanol. Cells were sonicated to release the virus and spun out at 8000 rpm for 10 minutes, 4°C. The supernatant was filtered through a 0.45 micron disc filter to remove particulates. The column was prepared for use by washing with 5ml water and charging with 0.5ml 0.1M nickel salt solution (supplied with kit); this was followed by a 5ml water wash. The column was equilibrated with 10ml of the lysis buffer prior to loading. The sample was applied to the column at a flow rate of 1ml per minute. Next, the column was washed with 10ml of lysis buffer. Fractions were eluted with a buffer composed of 1X Phosphate, 500mM Imidazole, 10% NP-40, 0.01M Mercaptoethanol in 1ml aliquots. Fractions were tested by Western Blot using an ECL kit. The primary antiserum was Rabbit anti Rat Cytomegalovirus IE1 and IE2 specific serum from Gordon Sandford, Johns Hopkins at a 1:300 dilution in PBS containing 1% Tween (such serum can be generated by the skilled artisan from isolation of native IE1 and IE2). The conjugate used was an HRP swine anti rabbit (DAKO) at 1:1000. Positive fractions were pooled and dialyzed against PBS (Spectra/Por 1 6,000-8,000 dialysis membrane). Protein determinations were made using the BCA microtiter plate method and samples were examined for purity by Coomassie Blue stain and Western Blot.

Figures 15A and B, respectively, show Western Blot and Coomassie Blue stained gel. In Figure 15A: lane 1 = SF9 insect cell lysate, lane 2= baculovirus RCMVIE1

infected SF9 cell lysate, lane 3= RCMVIE1 purified protein preparation, lane 4 = baculovirus RCMVIE2 infected SF9 cell lysate, lane 5 = RK-13 cells, lane 6 = vP1479 infected RK-13 cell lysate, and lane 7 = prestained molecular weight markers. In Figure 15B: lane 1 = RCMVIE1 purified protein preparation, and lane 2 = prestained molecular weight markers.

EXAMPLE 7 - ADDITIONAL BACULOVIRUS RECOMBINANTS

By employing the techniques of Smith et al., U.S. Patent No. 4,745,051, incorporated herein by reference, or of other literature concerning baculovirus recombinants, including the techniques of Example 6, with exogenous DNA of any of U.S. Patents Nos. 5,047,320, 5,075,213, Paoletti, U.S. Patent No. 5,338,683, Paoletti et al., U.S. Patent No. 5,494,807, Paoletti et al., PCT publication WO 96/39491, based on U.S. applications Serial Nos. 08/471,014, filed June 6, 1995, and 08/658,665, filed June 5, 1995 (see Example 3), Paoletti et al. WO 94/16716 based on U.S. applications Serial Nos. 007,115, filed January 21, 1993, and 184,009, filed January 19, 1994 (see Example 4), or other documents cited and incorporated herein, or literature concerning HCMV antigens, epitopes of interest, p53, p53 epitopes of interest, and DNA coding therefor, baculovirus embodiments expressing any desired HCMV and/or p53 epitope of interest, including those set forth in Examples 3 and 4 for various HCMV epitopes of interest and p53 epitopes of interest, and gene products therefrom, are obtained, for practice of this invention. Analysis confirms expression.

EXAMPLE 8 - ADENOVIRUS RECOMBINANTS

By employing the techniques of U.S. Patents Nos. 5,591,439 and 5,552,143, or of other literature concerning adenovirus recombinants with exogenous DNA of any of U.S. Patents Nos. 5,047,320, 5,075,213, Paoletti, U.S. Patent No. 5,338,683, Paoletti et al., U.S. Patent No. 5,494,807, Paoletti et al., PCT publication WO

96/39491, based on U.S. applications Serial Nos. 08/471,014, filed June 6, 1995, and 08/658,665, filed June 5, 1995 (see Example 3), Paoletti et al. WO 94/16716 based on U.S. applications Serial Nos. 007,115, filed January 21, 1993, and 184,009, filed January 19, 1994 (see Example 4), or other documents cited and incorporated herein, or literature concerning HCMV antigens, epitopes of interest, p53, p53 epitopes of interest, and DNA coding therefor, adenovirus embodiments expressing any desired HCMV and/or p53 epitope of interest, including the HCMV and p53 epitopes of interest of Examples 3 and 4 are obtained, for practice of this invention. Analysis confirms expression.

EXAMPLE 9 - DNA EXPRESSION SYSTEM EMBODIMENTS

By employing the techniques of U.S. Patents Nos. 5,591,639, 5,589,466, 5,580,589, incorporated herein by reference, or of other literature concerning DNA expression vectors with exogenous DNA of any of U.S. Patents Nos. 5,047,320, 5,075,213, Paoletti, U.S. Patent No. 5,338,683, Paoletti et al., U.S. Patent No. 5,494,807, Paoletti et al., PCT publication WO 96/39491, based on U.S. applications Serial Nos. 08/471,014, filed June 6, 1995, and 08/658,665, filed June 5, 1995 (see Example 3), Paoletti et al. WO 94/16716 based on U.S. applications Serial Nos. 007,115, filed January 21, 1993, and 184,009, filed January 19, 1994 (see Example 4), or other documents cited and incorporated herein or literature concerning HCMV antigens, epitopes of interest, p53, p53 epitopes of interest, and DNA coding therefor, DNA expression vector embodiments expressing any desired HCMV and/or p53 epitope of interest, including HCMV and p53 epitopes as in Examples 3 and 4 and gene products therefrom, are obtained, for practice of this invention. Analysis confirms expression.

EXAMPLE 10 - FORMULATIONS AND USE

Native HCMV epitopes are obtained from cells infected with HCMV, and native p53 epitopes are also

obtained from cells wherein expression thereof is detected. Recombinant HCMV and p53 epitopes are obtained from recombinants expressing these products, e.g., as in the previous Examples. These proteins are admixed with carrier, diluent etc., as herein described in amounts as herein described to obtain formulations. Recombinants and DNA expression systems expressing HCMV epitopes and p53 epitopes are obtained, e.g., as in the previous Examples; and, these recombinants and DNA expression systems are admixed with carrier, diluent, etc., as herein described to obtain formulations. Patients are administered the formulations as herein described for the prevention and/or treatment of vascular disease such as atherosclerosis and/or restenosis, including in a manner analogous to gene therapy directed against SMC proliferation, as described in literature cited herein. Propensity towards or against such disease is determined using diagnostic methods as herein described.

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited by particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope thereof.

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WHAT IS CLAIMED IS:

1. A composition for therapy for restenosis and/or atherosclerosis comprising an agent for decreasing viral load of cytomegalovirus.

2. The composition of claim 1 wherein the agent is an immunological composition or vaccine against cytomegalovirus CMV comprising at least one epitope of interest of CMV and/or an expression system which expresses at least one epitope of interest of CMV.

3. The composition of claim 2 comprising the expression system which expresses at least one epitope of interest of CMV.

4. The composition of claim 3 wherein the expression system is an adenovirus, poxvirus or DNA plasmid expression system.

5. The composition of claim 2 comprising the at least one epitope of interest of CMV.

6. The composition of claim 5 wherein the at least one epitope of interest is from expression by at least one recombinant.

7. The composition of claim 6 wherein the recombinant is an adenovirus, poxvirus, baculovirus, or DNA plasmid expression system.

8. The composition of claim 2 further comprising at least one epitope of interest of p53.

9. The composition of claim 2 wherein the CMV is human CMV.

10. The composition of claim 2 wherein the epitope of interest is selected from IE1 and/or IE2 or a portion thereof; gB; gB with transmembrane deleted therefrom; gH; gL; pp150; pp65; IE1 with amino acids 2-32 deleted therefrom; IE1 with amino acids 292-319 deleted therefrom; IE1 exon 4 segment; gB and gH; gB and pp65; gB, gH and pp65; gB, gH, pp65 and IE1 exon 4 segment; gB, gH, pp65, pp150, and IE1 exon 4 segment; gB, gH, pp65 and pp150; gB, gH, gL, pp65, pp150 and IE1 exon 4 segment; and gB, gH, gL, pp65 and pp150; gp64; or portion of such

CMV antigens.

11. The composition of claim 10 further comprising a p53 epitope of interest.

12. A composition for prophylaxis against restenosis and/or atherosclerosis comprising an agent for decreasing viral load of cytomegalovirus.

13. The composition of claim 11 wherein the agent is an immunological composition or vaccine against cytomegalovirus CMV comprising at least one an epitope of interest of CMV and/or an expression system which expresses at least one epitope of interest of CMV.

14. The composition of claim 13 comprising the expression system which expresses at least one epitope of interest of CMV.

15. The composition of claim 14 wherein the expression system is an adenovirus, poxvirus or DNA plasmid expression system.

16. The composition of claim 13 comprising the at least one epitope of interest of CMV.

17. The composition of claim 16 wherein the at least one epitope of interest is from expression by at least one recombinant.

18. The composition of claim 17 wherein the recombinant is an adenovirus, poxvirus, baculovirus, or DNA plasmid expression system.

19. The composition of claim 13 further comprising at least one epitope of interest of p53.

20. The composition of claim 13 wherein the CMV is human CMV.

21. The composition of claim 13 wherein the epitope of interest is selected from IE1 and/or IE2 or a portion thereof; gB; gB with transmembrane deleted therefrom; gH; gL; pp150; pp65; IE1 with amino acids 2-32 deleted therefrom; IE1 with amino acids 292-319 deleted therefrom; IE1 exon 4 segment; gB and gH; gB and pp65; gB, gH and pp65; gB, gH, pp65 and IE1 exon 4 segment; gB, gH, pp65, pp150, and IE1 exon 4 segment; gB, gH, pp65 and

pp150; gB, gH, gL, pp65, pp150 and IE1 exon 4 segment; and gB, gH, gL, pp65 and pp150; gp64; or portion of such CMV antigens.

22. The composition of claim 21 further comprising a p53 epitope of interest.

23. A method of treating restenosis comprising administering to a patient in need of such treatment, a composition as claimed in any one of claims 1 to 11.

24. A method of treating atherosclerosis comprising administering to a patient in need of such treatment, a composition as claimed in any one of claims 1 to 11.

25. A method of preventing restenosis comprising administering to a patient in need of such prevention, a composition as claimed in any one of claims 12 to 22.

26. A method of preventing atherosclerosis comprising administering to a patient in need of such prevention, a composition as claimed in any one of claims 12 to 22.

27. The method of claim 24 further comprising administering additional treatment for reducing CMV viral load and/or for inhibiting smooth muscle cell proliferation.

28. The method of claim 25 further comprising administering additional treatment for reducing CMV viral load and/or for inhibiting smooth muscle cell proliferation.

29. The method of claim 26 further comprising administering an additional agent for reducing CMV viral load and/or for inhibiting smooth muscle cell proliferation.

30. The method of claim 27 further comprising administering an additional agent for reducing CMV viral load and/or for inhibiting smooth muscle cell proliferation.

31. A method for diagnosing susceptibility to

atherosclerosis and/or restenosis, comprising screening a sample from a patient for antibodies to CMV and/or CMV proteins.

32. The method of claim 31 comprising screening a sample from a patient for specific viral proteins that predict whether CMV has been reactivated and/or antibodies thereto.

33. The method of claim 32 wherein the proteins are IE72, IE84, IE55.

34. The method of claim 31 further comprising detecting whether CMV nucleic acid is present in peripheral blood monocytes (PBMCs) and/or detecting a cellular-mediated immune response to CMV peptides or proteins is present and/or HLA phenotyping and/or HLA genotyping.

35. The method of claim 34 wherein the detecting CMV nucleic acid is mRNA and the detection is by PCR.

36. The method of claim 34 wherein detecting a cellular-mediated response comprises detecting whether PBMCs recognize and/or respond to CMV peptides or proteins.

37. The method of claim 34 wherein detecting a cellular-mediated response comprises administering a CMV skin test by administering CMV proteins or peptides intradermally or subcutaneously or intramuscularly and ascertaining the result of the skin test.

38. The method of claim 36 wherein the detecting whether PBMCs recognize and/or respond to CMV peptides or proteins comprises presenting CMV proteins or peptides to a patient's PBMCs and measuring either the proliferative response of the cells (PMBCs) or the cytokine profile.

39. The method of claim 31 further comprising screening a sample from a patient p53.

40. A method for diagnosing susceptibility to atherosclerosis and/or restenosis, comprising screening a

sample from a patient for specific viral proteins that predict whether CMV has been reactivated and/or antibodies thereto.

41. The method of claim 40 wherein the proteins are IE72, IE84, or IE55.

42. A method for diagnosing susceptibility to atherosclerosis and/or restenosis, comprising detecting whether CMV nucleic acid is present in peripheral blood monocytes (PBMCs) and/or detecting a cellular-mediated immune response to CMV peptides or proteins is present and/or HLA phenotyping and/or HLA genotyping.

43. The method of claim 42 wherein the method comprises detecting whether CMV nucleic acid is present in peripheral blood monocytes (PBMCs).

44. The method of claim 43 wherein the detecting CMV nucleic acid is mRNA and the detection is by PCR.

45. The method of claim 42 wherein the method is for detecting a cellular-mediated immune response to CMV peptides or proteins is present.

46. The method of claim 42 wherein detecting a cellular-mediated response comprises detecting whether PBMCs recognize and/or respond to CMV peptides or proteins.

47. The method of claim 45 wherein detecting a cellular-mediated response comprises administering a CMV skin test by administering CMV proteins or peptides intradermally or subcutaneously or intramuscularly and ascertaining the result of the skin test.

48. The method of claim 46 wherein the detecting whether PBMCs recognize and/or respond to CMV peptides or proteins comprises presenting CMV proteins or peptides to a patient's PBMCs and measuring either the proliferative response of the cells (PMBCs) or the cytokine profile.

49. A composition for therapy for restenosis and/or atherosclerosis comprising at least one epitope of

p53 or an expression system which expresses the epitope.

50. The composition of claim 49 comprising the expression system which expresses the epitope of p53.

51. The composition of claim 50 wherein the expression system is an adenovirus, poxvirus or DNA plasmid expression system.

52. The composition of claim 49 comprising the epitope of interest of p53.

53. The composition of claim 52 wherein the epitope is from expression by at least one recombinant.

54. The composition of claim 53 wherein the recombinant is an adenovirus, poxvirus, baculovirus, or DNA plasmid expression system.

55. A composition for prevention of restenosis and/or atherosclerosis comprising at least one epitope of p53 or an expression system which expresses the epitope.

56. The composition of claim 55 comprising the expression system which expresses the epitope of p53.

57. The composition of claim 56 wherein the expression system is an adenovirus, poxvirus or DNA plasmid expression system.

58. The composition of claim 55 comprising the epitope of interest of p53.

59. The composition of claim 58 wherein the epitope is from expression by at least one recombinant.

60. The composition of claim 59 wherein the recombinant is an adenovirus, poxvirus, baculovirus, or DNA plasmid expression system.

61. A method of treating restenosis comprising administering to a patient in need of such treatment, a composition as claimed in any one of claims 49 to 54.

62. A method of treating atherosclerosis comprising administering to a patient in need of such treatment, a composition as claimed in any one of claims 49 to 54.

63. A method of preventing restenosis comprising administering to a patient in need of such

prevention, a composition as claimed in any one of claims 55 to 60.

64. A method of preventing atherosclerosis comprising administering to a patient in need of such prevention, a composition as claimed in any one of claims 55 to 60.

65. The method of claim 62 further comprising administering additional treatment for reducing CMV viral load and/or for inhibiting smooth muscle cell proliferation.

66. The method of claim 63 further comprising administering additional treatment for reducing CMV viral load and/or for inhibiting smooth muscle cell proliferation.

68. The method of claim 64 further comprising administering an additional agent for reducing CMV viral load and/or for inhibiting smooth muscle cell proliferation.

69. The method of claim 65 further comprising administering an additional agent for reducing CMV viral load and/or for inhibiting smooth muscle cell proliferation.

70. A method for stratification of atherosclerosis and/or restenosis risk factors comprising detecting antibodies to CMV and/or CMV proteins and/or detecting whether CMV nucleic acid is present in peripheral blood monocytes (PBMCs) and/or detecting a cellular-mediated immune response to CMV peptides or proteins is present and/or HLA phenotyping and/or HLA genotyping.

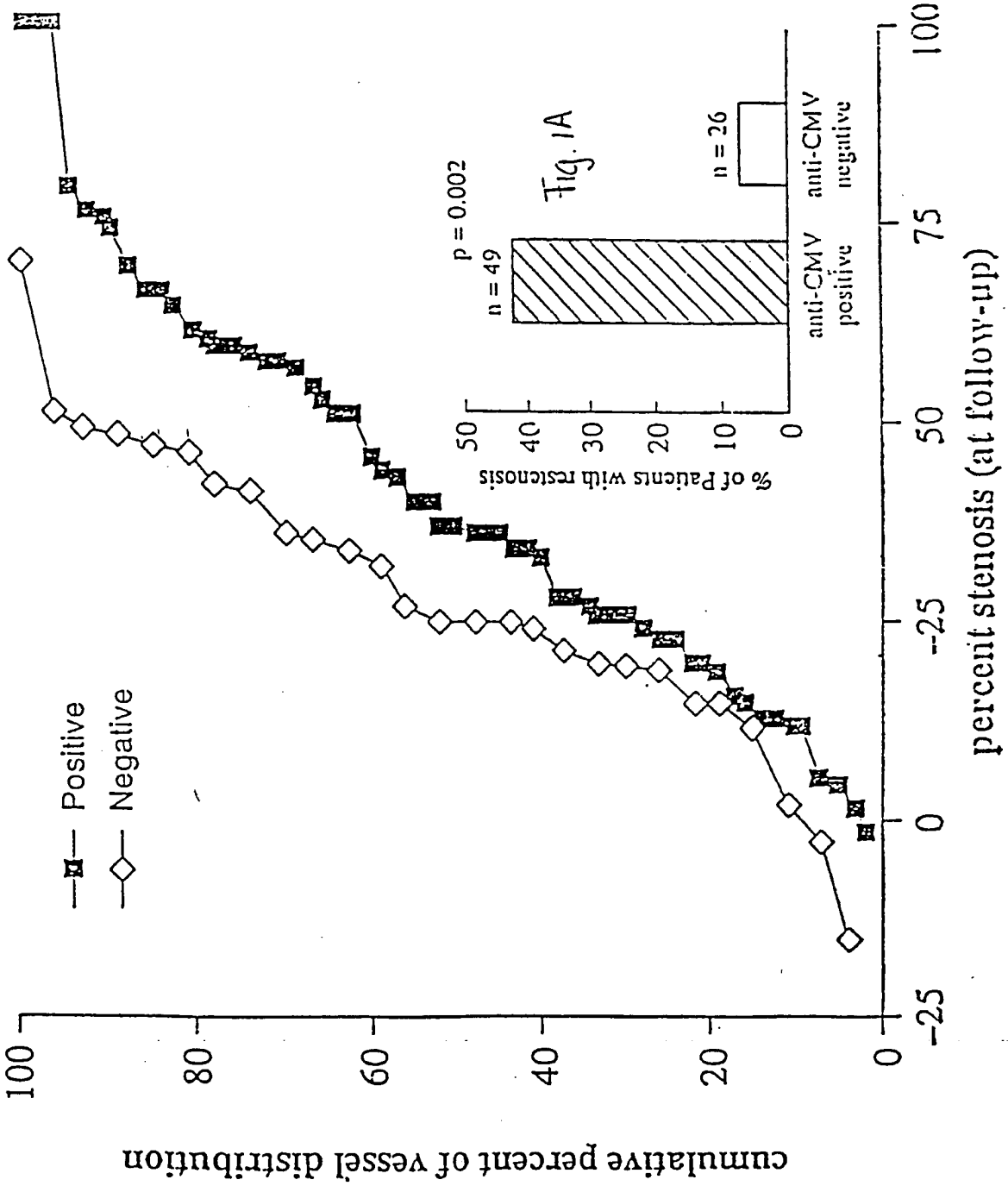

cumulative percent of vessel distribution
100
80
60
40
20
0
Positive
Negative
-25
0
25
50
75
100
percent stenosis (at follow-up)
% of Patients with restenosis
50
40
30
20
10
0
p = 0.002
n = 49
n = 26
anti-CMV positive
anti-CMV negative

Fig. 1A

Fig. 1

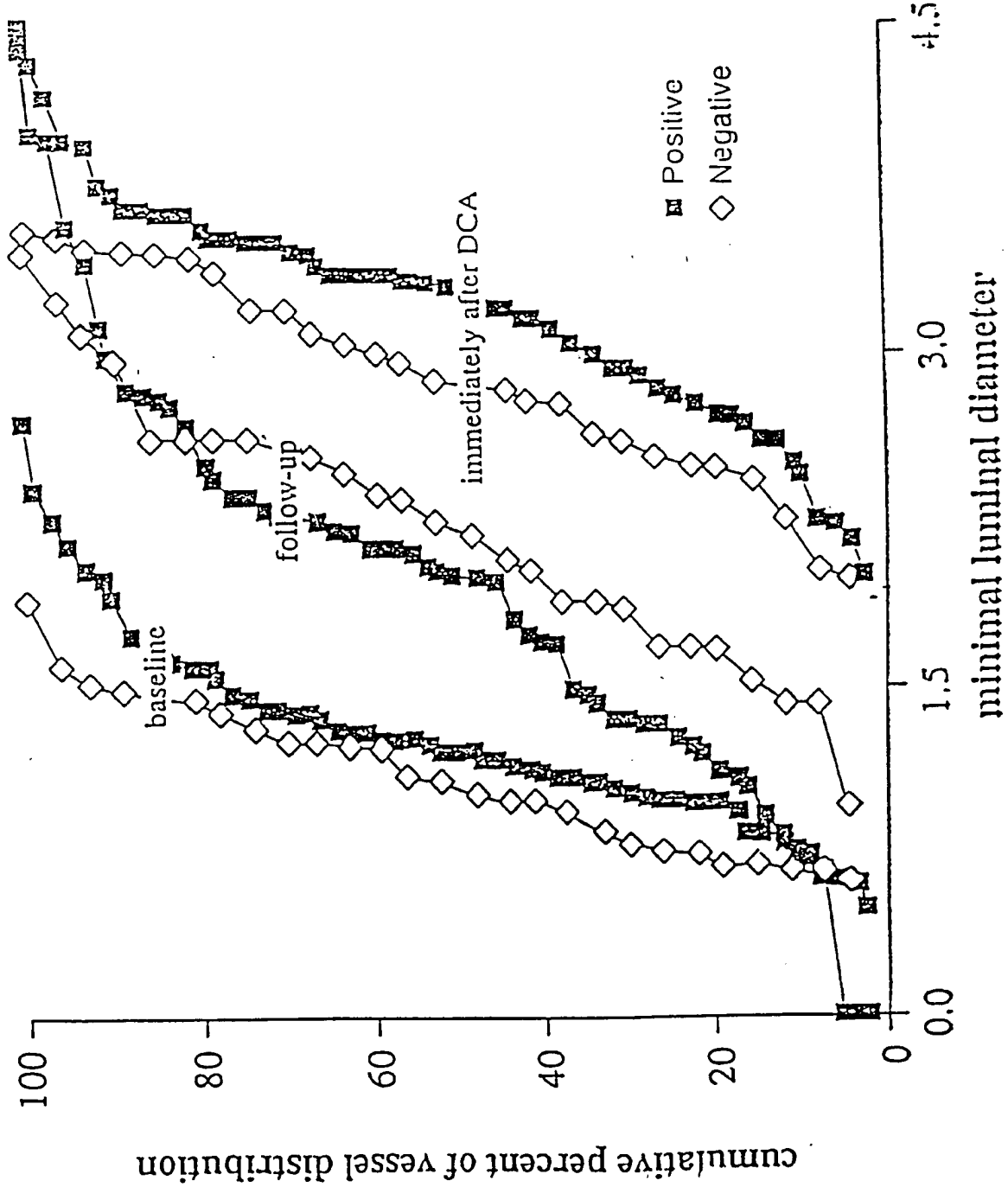

cumulative percent of vessel distribution
100
80
60
40
20
0
0.0
1.5
3.0
4.5
minimal luminal diameter
Positive
Negative
immediately after DCA
follow-up
baseline

Fig. 2

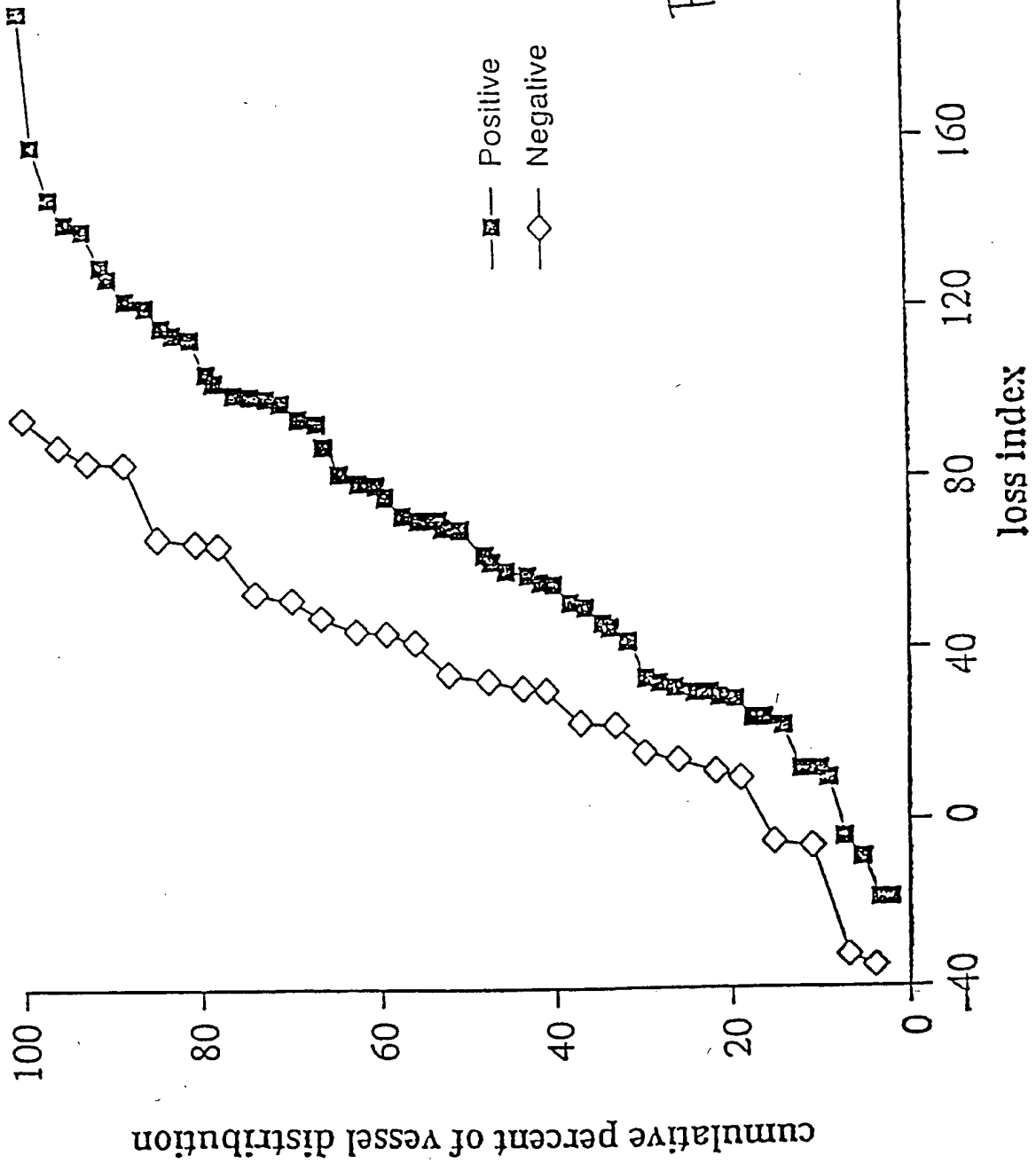

cumulative percent of vessel distribution
100
80
60
40
20
0
-40
0
40
80
120
160
200
loss index
Positive
Negative

Fig. 3

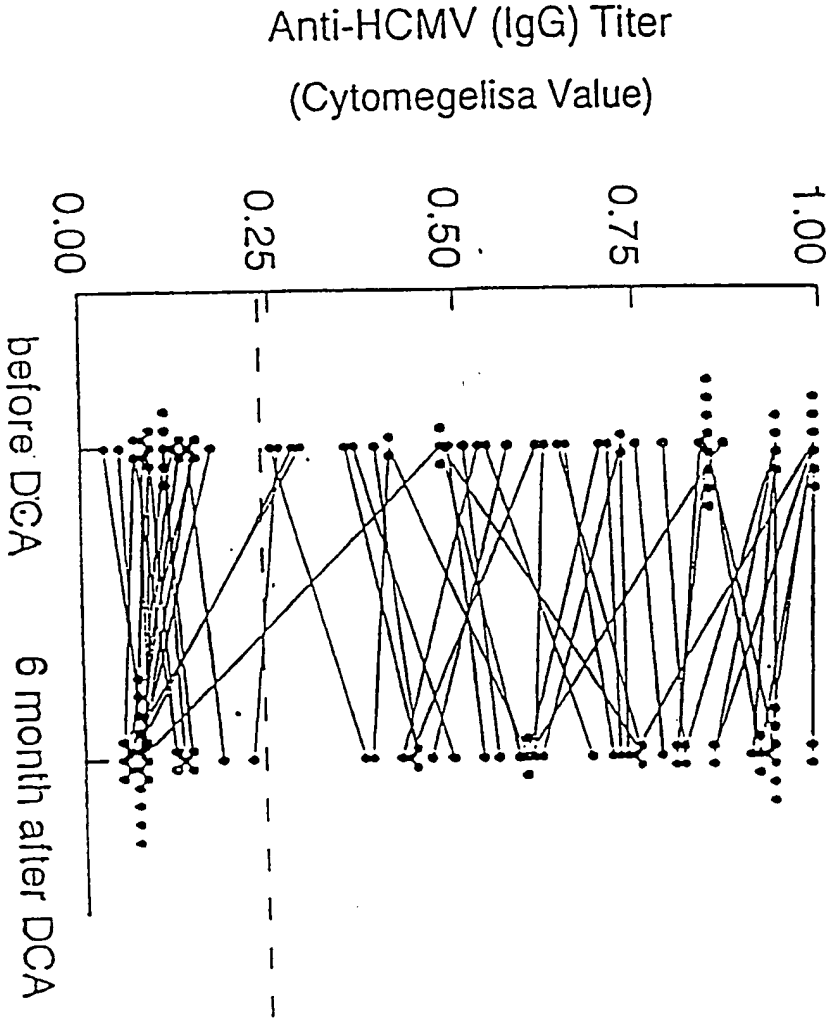

Anti-HCMV (IgG) Titer
(Cytomegelisa Value)
0.00
0.25
0.50
0.75
1.00
before DCA
6 month after DCA

Fig. 4

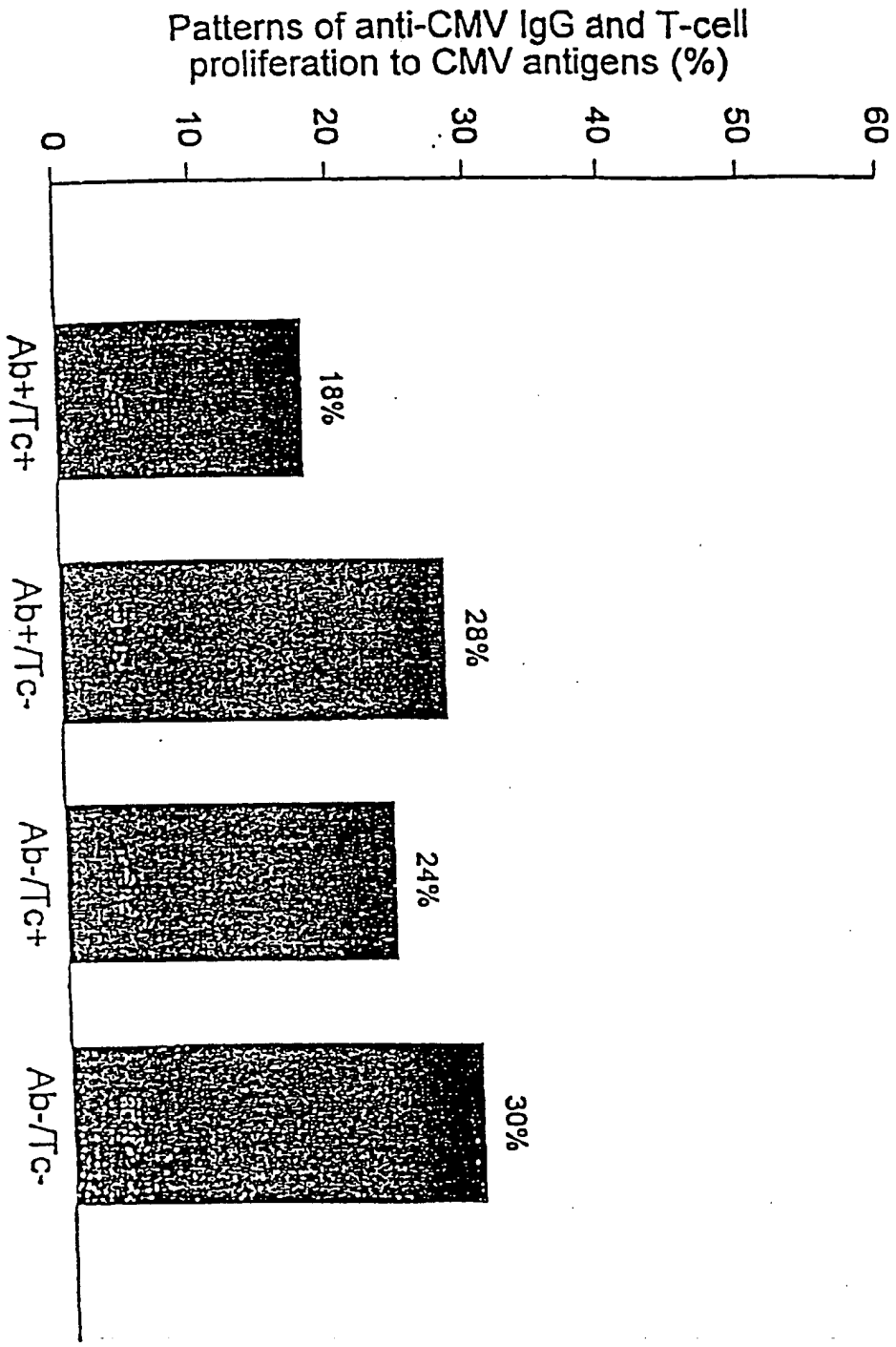

Patterns of anti-CMV IgG and T-cell proliferation to CMV antigens (%)
0
10
20
30
40
50
60
Ab+/Tc+
18%
Ab+/Tc-
28%
Ab-/Tc+
24%
Ab-/Tc-
30%

Fig. 5

Fig. 6A

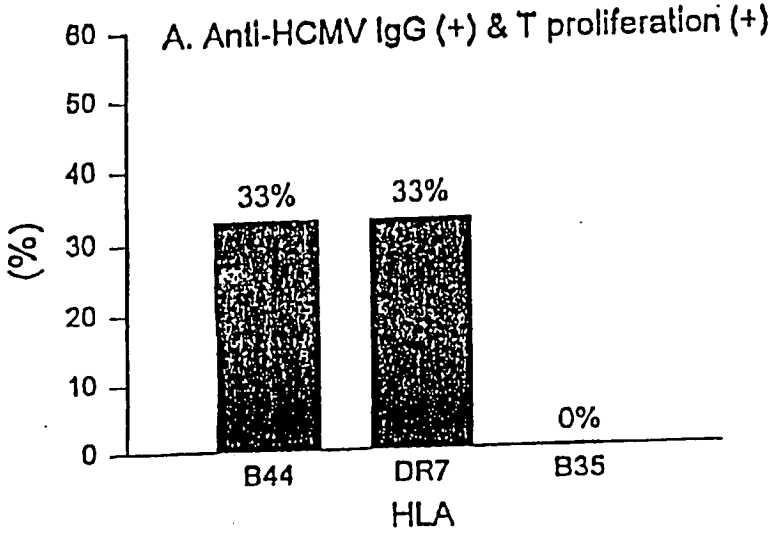
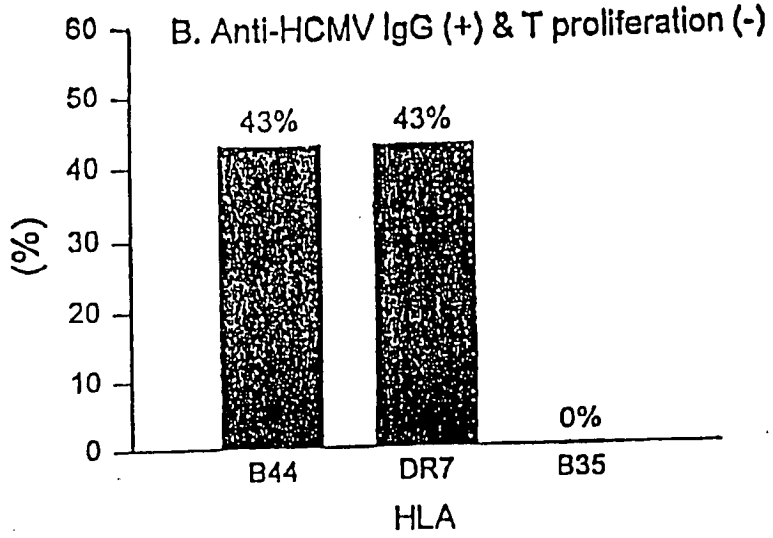


Fig. 6B



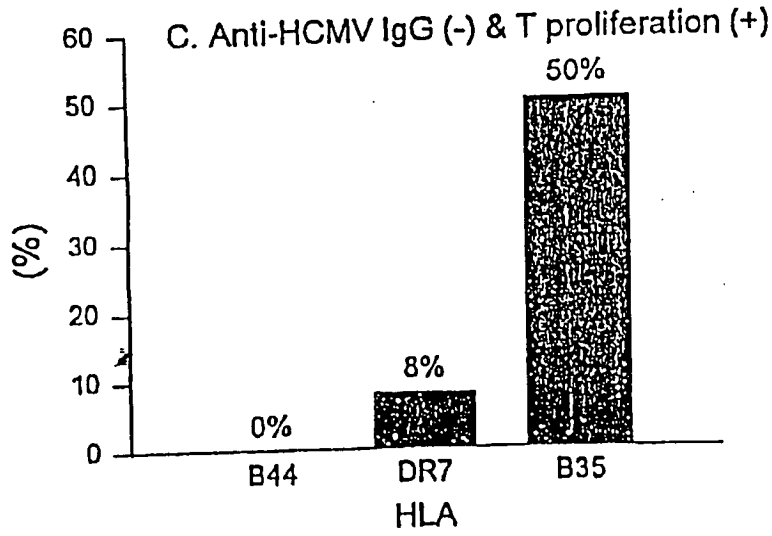


Fig. 6C

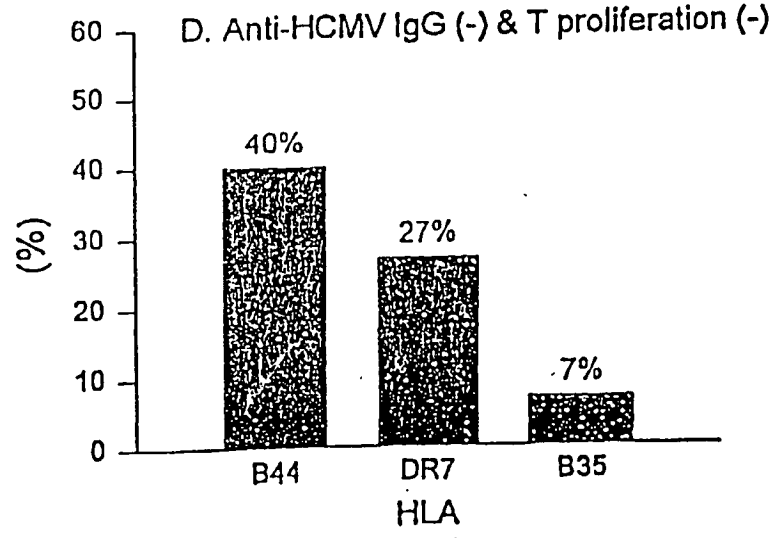


Fig. 6D

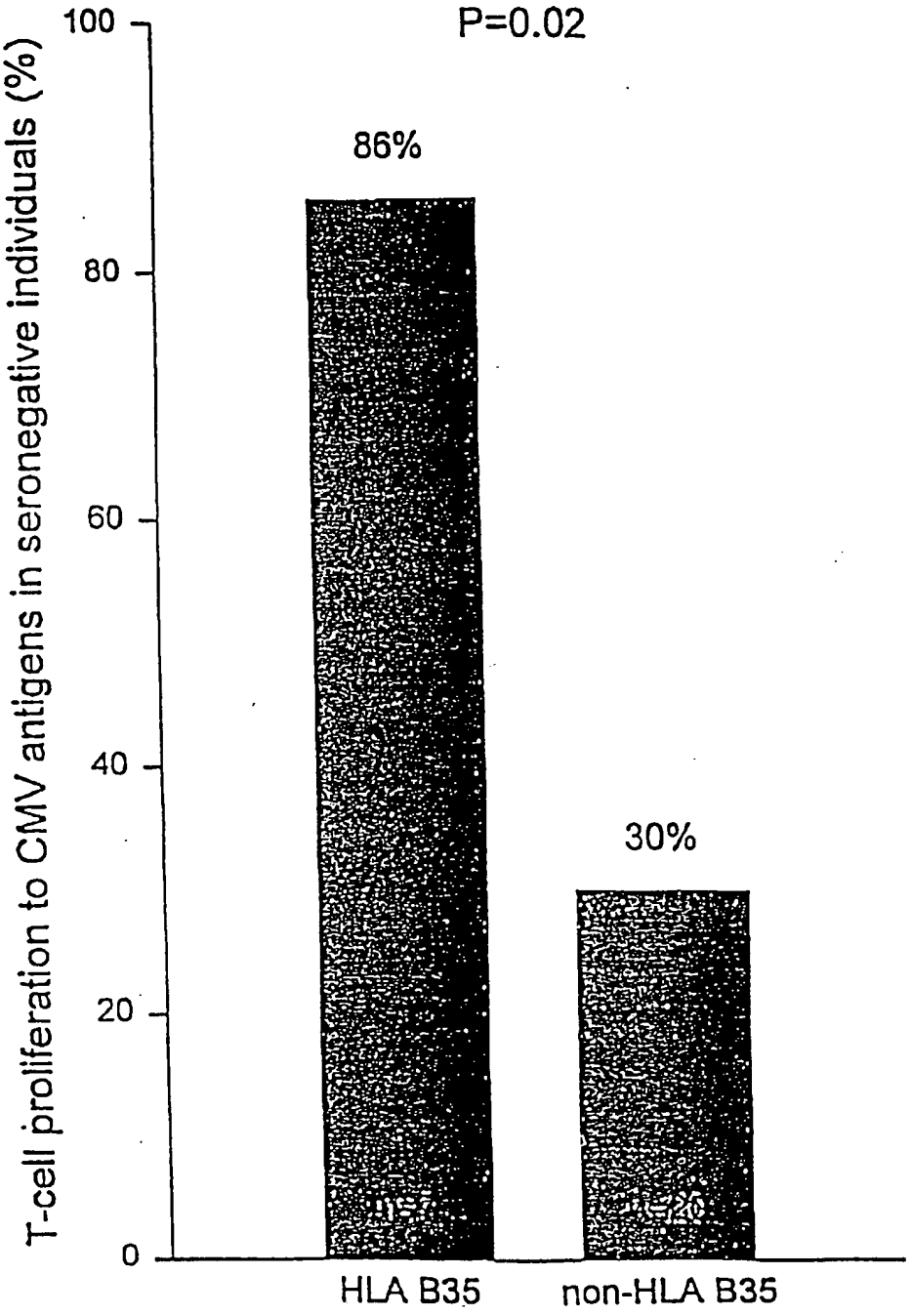

P=0.02
86%
30%
T-cell proliferation to CMV antigens in seronegative individuals (%)
100
80
60
40
20
0
HLA B35
non-HLA B35

Fig. 7

```
   1 TATAAATCTC CTAACGCCGT TCGGGCAGTC ACAGTCATCG GATCGGACGC CGTGGAACGC
  61 AGTTCTCAGC GAAGAAGGAC ACCGCCCGAC TCCAGAAGAC ACCGCTGCCC GAAGAAGAGA
 121 AGACTTCATC GGTAAGAGAC CCAGCTTCTC CTCCCCGGAG CTTCGGCCAC GCCGCTCCAC
 181 ACCCGGGAAC CGAGGCTTCG GAGCCCGATA CCCGGACAGA AGCTTCTCCC CGGCCGCTCC
 241 ACATCAGGGA GCCTTGACCG GCGAGCCTGC TATCCGGGTA GAGACTGTCC TGCGGCCGCT
 301 TCAGCAGCTC CACGATCGAC GACTGTGACC GTTGAGCCCG CCGTTTAGGC AGAGGCTCCG
 361 CTTCAACTAC CCTACCGACA CATTCGCGGT TCTTCCTCCA GAACATCTTA CCCTCTACTC
 421 GGCCACTCTA CAAGGACCGG TAATGGATCC AACTCTTTTC ACACAATCAA GACTTCTCAG
 481 AGTGAATGAT TATGATGAAG TGCGTGAGTC GGTAAATCAA CCGAGACAGG AACAGCAGCC
 541 AGGAGACAGG TGCCCTAGAC ATGTGGCAAG AATCATTGCC GAGAACGATC CTCCAATCAG
 601 ATGTGACCTG ACTCTCCAAG AGCTATTGAG TGAGGTGCAG GTGGATTTCG AACCATCGGC
 661 ATCAGAGGTC GTGGCAATGG AAGGCCTGAT GGACGAACAA CACTTCATTC CACATGATCC
 721 ACATTCTAAA AAAGCAGCCG TTCAAAGTCT TGTAATTGCC ATCAAGACCG CGGACCTCCT
 781 GTTGCAAATG ATACATGAGA ATGTTAAAAG AGACATCCGC ACGACATGCA TCCAAATGGC
 841 TAATGAATCT TATGCACGTG CGGACATAGT CAGAGATTCA CTGATAGCAG CATCGCAAGG
 901 AAAATACACA GCACTCGGGA AAATAGTATT CCACTCCTAT ACAAATTTCA TGCCAGTGAA
 961 TGCAAATGAG TCCGAAAAGA GAGCATGGAT GGAAATGCTA GGCGAGTGTA CCAGCCATGG
1021 AAACAAGCTG TGTGAGATGG CAAATGCGCA AGTAGAGCAG GAGACGCGCG ATATAATCAA
1081 TATAATGTTC AAAAATATAG ATGATGTAGT CACACAAACA ACAAGAGCAA TGAGAGGCGT
1141 GTTCGATCCA CCTGACACAG TTAAAGCTCT CTCTGCCGCA GCCCAACTGA TCAGAGTATG
1201 GGAACATGAT AACGTTATAA ATGACCAAAG TGTGTCAACA TCTTCTGTCG TAACGGCTGC
1261 ATTGGAGGCT AACGAGAATT TGGCAAAGGC ACTTAGAGAT GTGTCAGGGT ACGCTGAGGT
1321 GCAATTTAAC AGATTATGCC TTTCTATACT AACATCGGCA AAGGAACGAA TAGACATAAT
1381 CTATCATTCG GCAAGGTCCC AACACCTCGC GTGCAATGTC AGGATGAACG TGGCACAACA
1441 AAACCTAGCA ACTTTCATCC TAACGAATGC CAGAGAGAGG CCAAATGATG CTGTGATCAG
1501 AACACGCAGA GCAGTTGCAA ATACAGGTAT ACTGCTGTTC ACAGGACAAC ATATCACAAG
1561 AGATGCTTTA GATAAAGCTG CAGAGTCAAA AAGTGTAGAA GAAATTGTAG GGATGTCAGT
1621 ACAGGCTAGA CAAGCGCTAG TTGAACAAGA TATGCCTCCA CTAGAGGGAG AAGGTGAGGA
1681 AGCTAGAGAG GAACATGCCG GAGAAGGACA GGCTAGAGAA GGACAGGCCG AAGAAGAACA
1741 GGCCGGAGAA TCTGCGGGAG ATGAGTCCGA AGATGAAGAT GGCGAAGGAA GAAGGTCTCT
1801 GGTCCGTGTG ATCAACATTC CACTCGCGCA ACCTCAGCCG ATAGTGGCGC ACGAGCCTCC
1861 ACCTCAGCCC CAAGAATCGG ATGACAGCGA TACCGAATCT GATGGCGAAG ATCCAATCGC
1921 TAGGCAACAG AATCCCACAC CAACACAAGA GAGCGAACCC ATAACCGAAG ATCCTGAAGA
1981 CTGGCCGGAC GCTCAGAGAC TGATAGAAGA GGAATCTAGC CAAGAAACAC CCCAAGAACC
2041 GGCATCTGAG CAAGAACCAT CCACACCAGG TCCACGCACT AGGAGACGCT CACACCCCCC
2101 AACTGAAGGT TCAGCACCCA AGAGAGGCAG GAGATCATAA GGTGCCAACC AATATCAAAC
2161 CGATCGGGGT ACCAATCATA TAAATCATAA ATGCCAGGAT ACCAATCACA TAATCATATC
2221 AATATGCATC AATAAAATTT TATAATCATA CTCAGAGGGA ACTGCCCACC CTCAATTACC
2281 TATTGATTTT ACAATATATA ATGTAACTGC AATTAATAAA GTACACATGT ACATGA
```

Fig. 8

```
   1 TATAAATCTC  AAACGCCGT TCGGGCAGTC ACAGT..TCG GATCGGACGC CGTGGAACGC
  61 AGTTCTCAGC GAAGAAGGAC ACCGCCCGAC TCCAGAAGAC ACCGCTGCCC GAAGAAGAGA
 121 AGACTTCATC GGTAAGAGAC CCAGCTTCTC CTCCCCGGAG CTTCGGCCAC GCCGCTCCAC
 181 ACCCGGGAAC CGAGGCTTCG GAGCCCGATA CCCGGACAGA AGCTTCTCCC CGGCCGCTCC
 241 ACATCAGGGA GCCTTGACCG GCGAGCCTGC TATCCGGGTA GAGACTGTCC TGCGGCCGCT
 301 TCAGCAGCTC CACGATCGAC GACTGTGACC GTTGAGCCCG CCGTTTAGGC AGAGGCTCCG
 361 CTTCAACTAC CCTACCGACA CATTCGCGGT TCTTCCTCCA GAACATCTTA CCCTCTACTC
 421 GGCCACTCTA CAAGGACCGG TAATGGATCC AACTCTTTTC ACACAATCAA GACTTCTCAG
 481 AGTGAATGAT TATGATGAAG TGCGTGAGTC GGTAAATCAA CCGAGACAGG AACAGCAGCC
 541 AGGAGACAGG TGCCCTAGAC ATGTGGCAAG AATCATTGCC GAGAACGATC CTCCAATCAG
 601 ATGTGACCTG ACTCTCCAAG AGCTATTGAG TGAGGTGCAG GTGGATTTCG AACCATCGGC
 661 ATCAGAGGTC GTGGCAATGG AAGGCCTGAT GGACGAACAA CACTTCATTC CACATGATCC
 721 ACATTCTAAA AAAGCAGCCG GGCCTAACGT GAGGCATATA GACATTGTTA CCGCAGCCGC
 781 GTCGATGTCA GGGATATCCG GATCAACAGA GAGACCATTA GATGATGGAC AGAGACCCTT
 841 AGCTGATGGA TGTTATAGCA AGAAACATAA GAAGCAGAAA CACAGCGAAC CTATAGACAC
 901 CAAGGTGCAC ATCCAACGGG GGGAGGAAAC AGACTCTGAT TCAGACTCAG ACACCGGTAA
 961 ATCACCGGGA TGCGATGAAA TATCTTTTTA CTTGTCCAGT GCTTCGGATG ATGAACATGG
1021 CAATGGGAAT CGTTCTGGGT TAGAAGGAAA TTGTAGTTCA TATACTTCAC ATTCATCACG
1081 TAGATCAAAA TCGCCGCTAA GAAGTCCTTC AAACAGGCCC CAAAAGAGAA AATTATGTAA
1141 GAATATGTTT ATTACAAAAA GCAAACGTAG GGTAATATGT GAATCTGATT CAGATACAGA
1201 CTCCGAAATC GAGACCAGGC CATTTATCAG ACCACAAGAA CCTCCCAGAC AAAAGAATAA
1261 GGGGAAAAGA TGTCCCAAGA AACATAGAAA GATAAAAGAG CTCATGGATG GGCCAGGATT
1321 CGTGGCTCCG AATGCACACA AACGAGGTAA AAATAGAAAT GAGGGAAACA ACGATGGACG
1381 AGGGAAACCG ACCACACGAG CTTTAGAATA CAAACAGATG CCATACAAAC AGCAAACGGT
1441 CCAGTTTCTC TATGGAAATG CGATAAGGAC ATGTAGAGAG AGCACCGTAC ACGATAAAAT
1501 TATTATGGTG ATGTTTACAC GGGGTCAAGA TATCAGGCAG GCCATAGAAA AGTTGAGATC
1561 CCAACTTGGT CAAATAACCA ACCTTTCCAT ATCTGCTCCC TTCAACACAG AACACACAAA
1621 ACCACAGATA CACACACCAA ACACGGTTAA CATGACATCG CAGGCACTTG CGGCAGGTCT
1681 TCAAGCCTCC TGGAACCTAG ACGAGGATAA TAAACACAAT AATGCACCTA GGATGTCAGA
1741 TTACAGAACC ATGATAATCC AAGCGGCAAC ACCACCAGAT TTTCTAGGTG CACTCAAACT
1801 ATGCATACAG TTCGCACAAA CCTTTCCCAA GAATGCGTGT ATAAGGTTAT GTAATATAGT
1861 TGGAGGCCTA CAACCCCTTC CCATCTACGA AAAAGTCGTC ACCGCTTACA CTGACACGCA
1921 ATATAACTTT AGCCCAATCA CTAACAAAGA TAGTAACGGT GGTATGAGCA CAATATTGGA
1981 TCAGGACTCC GATTCAGAAT AATGAAGAAA CTATCATATT AAATCGTGTA CATATTTTAT
2041 TAAACACTAT TTCCAACCAT GAGACGAGGC TTGTTGATGC AGCTGCTGTT CCTTGGAATA
2101 AATGTAATAT ACTGT
```

Fig. 9

```
   1 AAGCTTGCGG  GCTCATTA GACAAGCGAA TGAGGC..GA AAACGTGGAG GAGGTATTAA
  61 GTTTGGAGAA ATGGAGAGAG ACTGTTTAAT AGCGCATGGC GCAGCCAATA CTATTACAGA
 121 AGTTTTGAAA GATTCGGAAG AAGATTATCA AGATGTGTAT GTTTGTGAAA ATTGTGGAGA
 181 CATAGCAGCA CAAATCAAGG GTATTAATAC ATGTCTTAGA TGTTCAAAAC TTAATCTCTC
 241 TCCTCTCTTA ACAAAAATTG ATACCACGCA CGTATCTAAA GTATTTCTTA CTCAAATGAA
 301 CGCCAGAGGC GTAAAAGTCA AATTAGATTT CGAACGAAGG CCTCCTTCGT TTTATAAACC
 361 ATTAGATAAA GTTGATCTCA AGCCGTCTTT TCTGGTGTAA TAAAAATTAA TTAATTACTC
 421 GAGATAAAAA TTATGATCTC CTGCCTCTCT TGGGTGCTGA ACCTTCAGTT GGGGGGTGTG
 481 AGCGTCTCCT AGTGCGTGGA CCTGGTGTGG ATGGTTCTTG CTCAGATGCC GGTTCTTGGG
 541 GTGTTTCTTG GCTAGATTCC TCTTCTATCA GTCTCTGAGC GTCCGGCCAG TCTTCAGGAT
 601 CTTCGGTTAT GGGTTCGCTC TCTTGTGTTG GTGTGGGATT CTGTTGCCTA GCGATTGGAT
 661 CTTCGCCATC AGATTCGGTA TCGCTGTCAT CCGATTCTTG GGGCTGAGGT GGAGGCTCGT
 721 GCGCCACTAT CGGCTGAGGT TGCGCGAGTG GAATGTTGAT CACACGGACC AGAGACCTTC
 781 TTCCTTCGCC ATCTTCATCT TCGGACTCAT CTCCCGCAGA TTCTCCGGCC TGTTCTTCTT
 841 CGGCCTGTCC TTCTCTAGCC TGTCCTTCTC CGGCATGTTC CTCTCTAGCT TCCTCACCTT
 901 CTCCCTCTAG TGGAGGCATA TCTTGTTCAA CTAGCGCTTG TCTAGCCTGT ACTGACATCC
 961 CTACAATTTC TTCTACACTT TTTGACTCTG CAGCTTTATC TAAAGCATCT CTTGTGATAT
1021 GTTGTCCTGT GAACAGCAGT ATACCTGTAT TTGCAACTGC TCTGCGTGTT CTGATCACAG
1081 CATCATTTGG CCTCTCTCTG GCATTCGTTA GGATGAAAGT TGCTAGGTTT TGTTGTGCCA
1141 CGTTCATCCT GACATTGCAC GCGAGGTGTT GGGACCTTGC CGAATGATAG ATTATGTCTA
1201 TTCGTTCCTT TGCCGATGTT AGTATAGAAA GGCATAATCT GTTAAATTGC ACCTCAGCGT
1261 ACCCTGACAC ATCTCTAAGT GCCTTTGCCA AATTCTCGTT AGCCTCCAAT GCAGCCGTTA
1321 CGACAGAAGA TGTTGACACA CTTTGGTCAT TTATAACGTT ATCATGTTCC CATACTCTGA
1381 TCAGTTGGGC TGCGGCAGAG AGAGCTTTAA CTGTGTCAGG TGGATCGAAC ACGCCTCTCA
1441 TTGCTCTTGT TGTTTGTGTG ACTACATCAT CTATATTTTT GAACATTATA TTGATTATAT
1501 CGCGCGTCTC CTGCTCTACT TGCGCATTTG CCATCTCACA CAGCTTGTTT CCATGGCTGG
1561 TACACTCGCC TAGCATTTCC ATCCATGCTC TCTTTTCGGA CTCATTTGCA TTCACTGGCA
1621 TGAAATTTGT ATAGGAGTGG AATACTATTT TCCCGAGTGC TGTGTATTTT CCTTGCGATG
1681 CTGCTATCAG TGAATCTCTG ACTATGTCCG CACGTGCATA AGATTCATTA GCCATTTGGA
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1861 GAATGAAGTG TTGTTCGTCC ATCAGGCCTT CCATTGCCAC GACCTCTGAT GCCGATGGTT
1921 CGAAATCCAC CTGCACCTCA CTCAATAGCT CTTGGAGAGT CAGGTCACAT CTGATTGGAG
1981 GATCGTTCTC GGCAATGATT CTTGCCACAT GTCTAGGGCA CCTGTCTCCT GGCTGCTGTT
2041 CCTGTCTCGG TTGATTTACC GACTCACGCA CTTCATCATA ATCATTCACT CTGAGAAGTC
2101 TTGATTGTGT GAAAAGAGTT GGATCCATTA CGATACAAAC TTAACGGATA TCGCGATAAT
2161 GAAATAATTT ATGATTATTT CTCGCTTTCA ATTTAACACA ACCCTCAAGA ACCTTTGTAT
2221 TTATTTTCAC TTTTTAAGTA TAGAATAAAG AAGAATTGGG TTTTGGGATT TCAAAATTGA
2281 AAATATATAA TTACAATATA AAATGGATCC AACTCTTTTC ACACAATCAA GACTTCTCAG
2341 AGTGAATGAT TATGATGAAG TGCGTGAGTC GGTAAATCAA CCGAGACAGG AACAGCAGCC
2401 AGGAGACAGG TGCCCTAGAC ATGTGGCAAG AATCATTGCC GAGAACGATC CTCCAATCAG
2461 ATGTGACCTG ACTCTCCAAG AGCTATTGAG TGAGGTGCAG GTGGATTTCG AACCATCGGC
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2581 ACATTCTAAA AAAGCAGCCG GGCCTAACGT GAGGCATATA GACATTGTTA CCGCAGCCGC
2641 GTCGATGTCA GGGATATCCG GATCAACAGA GAGACCATTA GATGATGGAC AGAGACCCTT
2701 AGCTGATGGA TGTTATAGCA AGAAACATAA GAAGCAGAAA CACAGCGAAC CTATAGACAC
2761 CAAGGTGCAC ATCCAACGGG GGGAGGAAAC AGACTCTGAT TCAGACTCAG ACACCGGTAA
2821 ATCACCGGGA TGCGATGAAA TATCTTTTTA CTTGTCCAGT GCTTCGGATG ATGAACATGG
2881 CAATGGGAAT CGTTCTGGGT TAGAAGGAAA TTGTAGTTCA TATACTTCAC ATTCATCACG
2941 TAGATCAAAA TCGCCGCTAA GAAGTCCTTC AAACAGGCCC CAAAAGAGAA AATTATGTAA
3001 GAATATGTTT ATTACAAAAA GCAAACGTAG GGTAATATGT GAATCTGATT CAGATACAGA
3061 CTCCGAAATC GAGACCAGGC CATTTATCAG ACCACAAGAA CCTCCCAGAC AAAAGAATAA
```
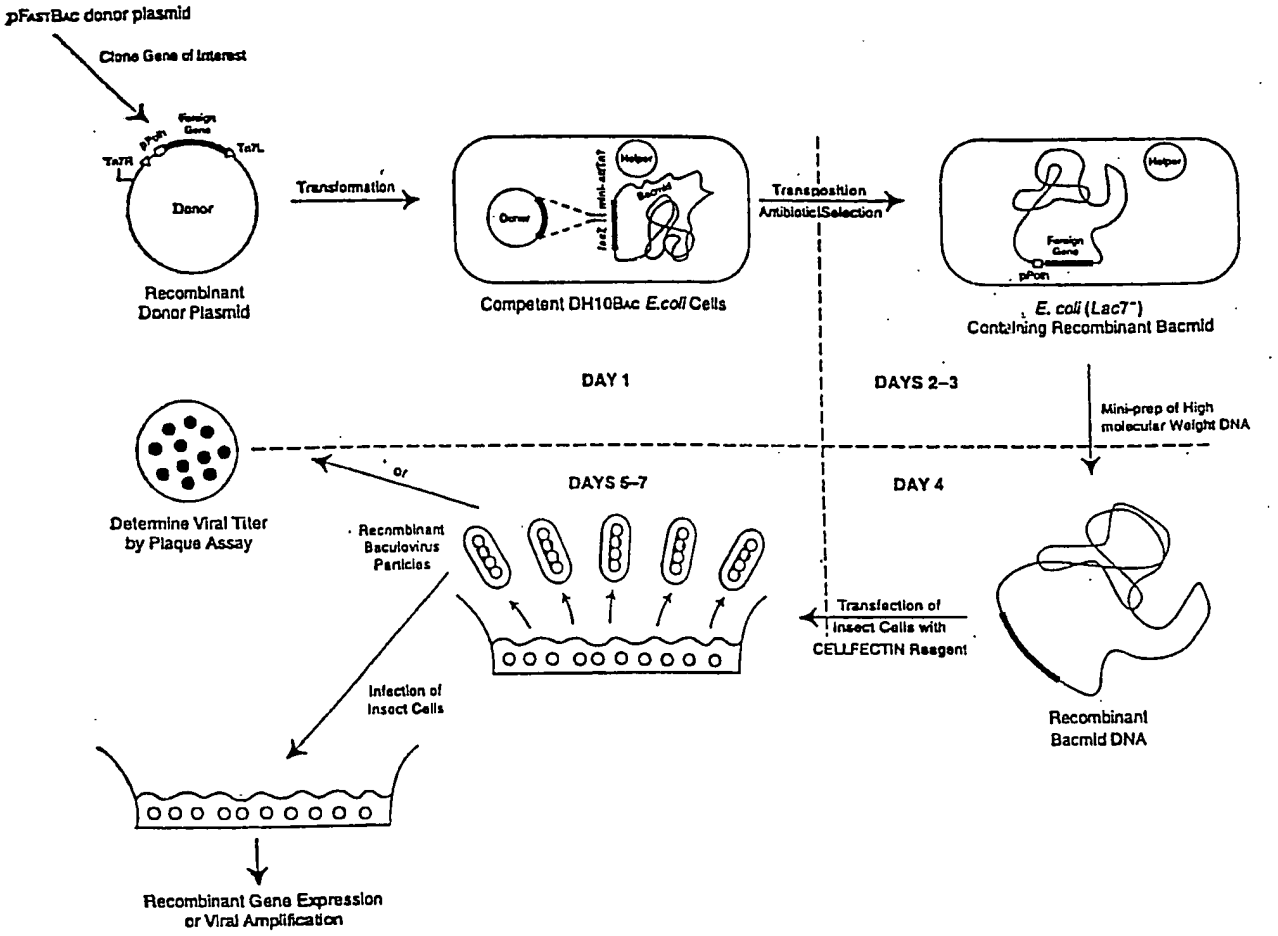

Fig. 10A

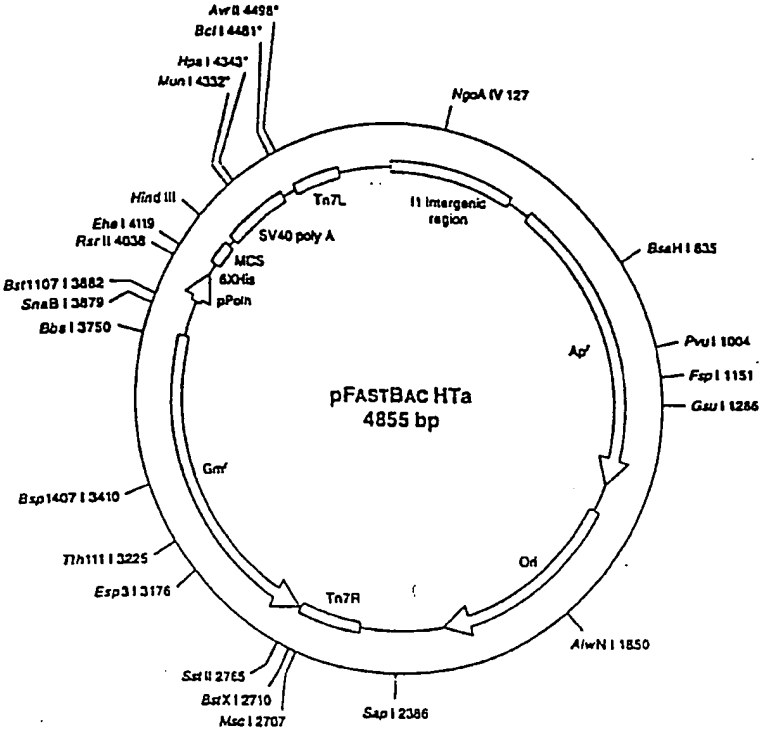
```
3121 GGGGAAAAGA  GTCCCAAGA AACATAGAAA GATA  GAG CTCATGGATG GGCCAGGATT
3181 CGTGGCTCCG AATGCACACA AACGAGGTAA AAATAGAAAT GAGGGAAACA ACGATGGACG
3241 AGGGAAACCG ACCACACGAG CTTTAGAATA CAAACAGATG CCATACAAAC AGCAAACGGT
3301 CCAGTTTCTC TATGGAAATG CGATAAGGAC ATGTAGAGAG AGCACCGTAC ACGATAAAAT
3361 TATTATGGTG ATGTTTACAC GGGGTCAAGA TATCAGGCAG GCCATAGAAA AGTTGAGATC
3421 CCAACTTGGT CAAATAACCA ACCTTTCCAT ATCTGCTCCC TTCAACACAG AACACACAAA
3481 ACCACAGATA CACACACCAA ACACGGTTAA CATGACATCG CAGGCACTTG CGGCAGGTCT
3541 TCAAGCCTCC TGGAACCTAG ACGAGGATAA TAAACACAAT AATGCACCTA GGATGTCAGA
3601 TTACAGAACC ATGATAATCC AAGCGGCAAC ACCACCAGAT TTTCTAGGTG CACTCAAACT
3661 ATGCATACAG TTCGCACAAA CCTTTCCCAA GAATGCGTGT ATAAGGTTAT GTAATATAGT
3721 TGGAGGCCTA CAACCCCTTC CCATCTACGA AAAAGTCGTC ACCGCTTACA CTGACACGCA
3781 ATATAACTTT AGCCCAATCA CTAACAAAGA TAGTAACGGT GGTATGAGCA CAATATTGGA
3841 TCAGGACTCC GATTCAGAAT AATTTTTATC GCGATAGCTG ATTAGTTTTT GTTAACAAAA
3901 ATGTGGGAGA ATCTAATTAG TTTTTCTTTA CACAATTGAC GTACATGAGT CTGAGTTCCT
3961 TGTTTTTGCT AATTATTTCA TCCAATTTAT TATTCTTGAC GATATCGAGA TCTTTTGTAT
4021 AGGAGTCAGA CTTGTATTCA ACATGCTTTT CTATAATCAT CTTAGTTATT TCGGCATCAT
4081 CCAATAGTAC ATTTTCCAGA TTAACAGAGT AGATATTAAT GTCGTATTTG AACAGAGCCT
4141 GTAACATCTC AATGTCTTTA TTATCTATAG CCAATTTAAT GTCCGGAATG AAGAGAAGGG
4201 AATTATTGGT GTTTGTCGAC GTCATATAGT CGAGCAAGAG AATCATCATA TCCACGTGTC
4261 CATTTTTTAT AGTGGTGTGA ATACAACTAA GGAGAATAGC CAGATCAAAA GTAGATGGTA
4321 TTTCTGAAAG AAAGTATGAT ACAATACTTA CATCATTAAG CATGACGGCA TGATAAAATG
4381 AAGTTTTCCA TCCAGTTTTC CCATAGAACA TCAGTCTCCA ATTTTTCTTA AACAGTTTCA
4441 CCGTTTGCAT GTTACCACTA TCAACCGCAT AATACAATGC GGTGTTTCCT TTGTCATCAA
4501 ATTGTGAATC ATCCATTCCA CTGAATAGCA AAATCTTTAC TATTTTGGTA TCTTCTAATG
4561 TGGCTGCCTG ATGTAATGGA AATTCATTCT CTAGAAGATT TTTCAATGCT CCAGCGTTCA
4621 ACAACGTACA TACTAGACGC ACGTTATTAT CAGCTATTGC ATAATACAAG GCACTATGTC
4681 CATGGACATC CGCCTTAAAT GTATCTTTAC TAGAGAGAAA GCTTTTCAGC TGCTTAGACT
4741 TCCAAGTATT AATTCGTGAC AGATCCATGT CTGAAACGAG ACGCTAATTA GTGTATATTT
4801 TTTCATTTTT TATAATTTTG TCATATTGCA CCAGAATTAA TAATATCTCT AATAGATCTA
4861 ATTTAATTTA ATTTATATAA CTTATTTTTT GAATATACTT TTAATTAACA AAAGAGTTAA
4921 GTTACTCATA TGGACGCCGT CCAGTCTGAA CATCAATCTT TTTAGCCAGA GATATCATAG
4981 CCGCTCTTAG AGTTTCAGCG TGATTTTCCA ACCTAAATAG AACTTCATCG TTGCGTTTAC
5041 AACACTTTTC TATTTGTTCA AACTTTGTTG TTACATTAGT AATCTTTTTT TCCAAATTAG
5101 TTAGCCGTTG TTTGAGAGTT TCCTCATTGT CGTCTTCATC GGCTTTAACA ATTGCTTCGC
5161 GTTTAGCCTC CTGGCTGTTC TTATCAGCCT TTGTAGAAAA AAATTCAGTT GCTGGAATTG
5221 CAAGATCGTC ATCTCCGGGG AAAAGAGTTC CGTCCATTTA AAGCCGCGGG AATTC
```

Fig. 10B



Generation of recombinant baculoviruses and gene expression with the Bac-to-Bac Expression System. The gene of interest is cloned into a pFastBac donor plasmid, and the recombinant plasmid is transformed into DH10Bac competent cells which contain the bacmid with a mini-*att*Tn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac donor plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by disruption of the *lacZ*α gene. High molecular weight mini-prep DNA is prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA is then used to transfect insect cells.

Fig. 11



Map and restriction endonuclease sites for pFastBac HT expression vectors. The circle map of pFastBac HTa (4855 bp) is presented. A similar map can be drawn for pFastBac HTb (4856 bp) and pFastBac HTc (4857 bp) except that the starred restriction endonucleases are shifted by +1 and +2 bases respectively.

Restriction endonucleases that do not cleave pFastBac HT DNAs:

Aat II	*Bpu*1102 I	*Bst*E II	*Eco*72 I	*Nhe* I	*Pfl*M I	*Pvu* II	*Sma* I	*Sun* II
Afl II	*Bsg* I	*Cla* I	*Eco*O109 I	*Nru* I	*Pin*A I	*Sex*A I	*Srf* I	*Swa* I
Apa I	*Bsp*M I	*Cvn* I	*Mlu* I	*Nsi* I	*Pme* I	*Sfi* I	*Sse* I	*Xcm* I
Asc I	*Bss*H II	*Eco*47 III	*Nde* I	*Pac* I	*Psp*5 II	*Sgr*A I	*Sse*8387 I	

Restriction endonucleases that cleave pFastBac HTa DNA twice:

Acc I	3882	4150	*BsaH* I	835	4119	*Rca* I	536	1544
Afl III	2264	3246	*Bsm* I	4327	4426	*Sca* I	893	4230
Ban II	157	4156	*Dra* III	230	3578	*Tfi* I	2290	4181
Bgl II	2547	3017	*Eam*1105 I	1371	4731	*Xmn* I	772	3797
Bsa I	1304	3661	*Eco*R V	2823	4086			

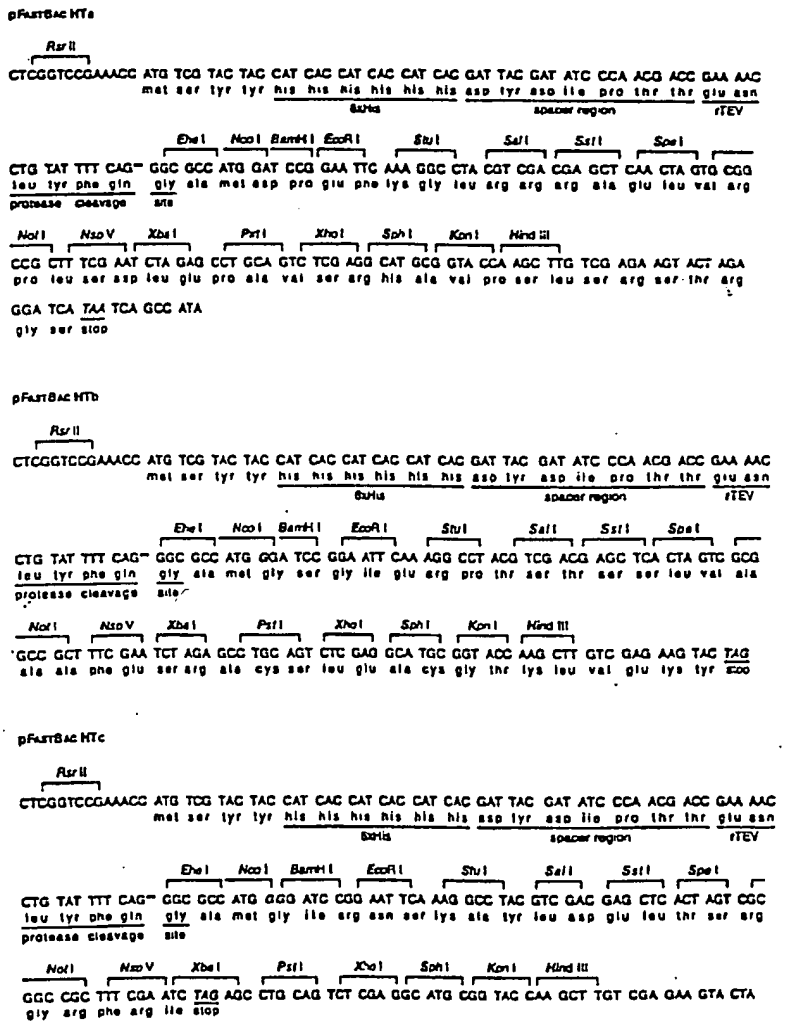
Restriction endonucleases that cleave pFastBac HTb DNA twice:

Acc I	3882	4151	*BsaH* I	835	4119	*Rca* I	536	1544
Afl III	2264	3246	*Bsm* I	4328	4427	*Sca* I	893	4231
Ban II	157	4157	*Dra* III	230	3578	*Tfi* I	2290	4182
Bgl II	2547	3017	*Eam*1105 I	1371	4732	*Xmn* I	772	3797
Bsa I	1304	3661						

Restriction endonucleases that cleave pFastBac HTc DNA twice:

Acc I	3882	4152	*Bsm* I	4329	4428	*Rca* I	536	1544
Afl III	2264	3246	*Dra* III	230	3578	*Sca* I	893	4232
Ban II	157	4158	*Eam*1105 I	1371	4733	*Tfi* I	2290	4183
Bsa I	1304	3661	*Eco*R V	2823	4086	*Xmn* I	772	3797
BsaH I	835	4119						

Fig. 12



Multiple cloning site sequences of pFastBac HT expression vectors. The multiple cloning sites (MCS) for the three vectors are shown above. The sequence for the 6xHis, spacer region and rTEV protease cleavage site are underlined. The cleavage with rTEV protease occurs between the gln and gly and is signified by **. The shift in reading frame occurs at the *Bam*H I site in each vector. The added base(s) are shown in bold. The stop codon for each vector is underlined and italicized. In pFastBac HTc the stop codon is within the MCS at the *Xba* I site. The 5' end of a gene must be cloned upstream of the *Xba* I site in pFastBac HTc to be translated.

Fig. 13

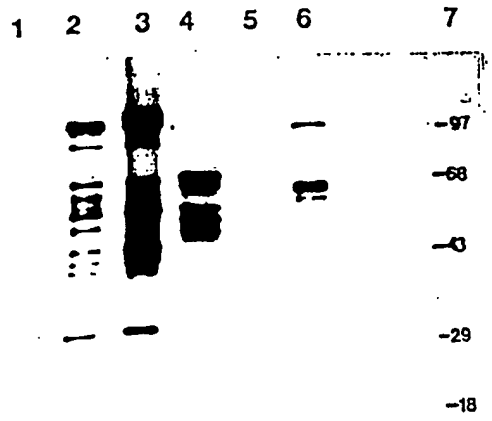
```
   1 ATGGAGTCCT  GCCAAGAG AAAGATGGAC CCTGA  ATC CTGACGAGGG CCCTTCCTCC
  61 AAGGTGCCAC GGCCCGAGAC ACCCGTGACC AAGGCCACGA CGTTCCTGCA GACTATGTTG
 121 AGGAAGGAGG TTAACAGTCA GCTGAGTCTG GGAGACCCGC TGTTTCCAGA GTTGGCCGAA
 181 GAATCCCTCA AAACTTTTGA ACAAGTGACC GAGGATTGCA ACGAGAACCC CGAGAAAGAT
 241 GTCCTGGCAG AACTCGGTGA CATCCTCGCC CAGGCTGTCA ATCATGCCGG TATCGATTCC
 301 AGTAGCACCG GCCCCACGCT GACAACCCAC TTCCGCAGCG TTAGACGCGC CCCTCTTAAC
 361 AAGCCGACCC CCACCAGCGT CGCGGTTACT AACACTCCTC TCCCCGGGGC ATCCGCTACT
 421 CCCGAGCTCA GCCCGCGTAA GAAACCGCGC AAAACCACGC GTCCTTTCAA GGTGATTATT
 481 AAACCGCCCG TGCCTCCCGC GCCTATCATG CTGCCCCTCA TCAAACAGGA AGACATCAAG
 541 CCCGAGCCCG ACTTTACCAT CCAGTACCGC AACAAGATTA TCGATACCGC CGGCTGTATC
 601 GTGATCTCTG ATAGCGAGGA AGAACAGGGT GAAGAAGTCG AAACCCGCGG TGCTACCGCG
 661 TCTTCCCCTT CCACCGGCAG CGGCACGCCG CGAGTGACCT CTCCCACGCA CCCGCTCTCC
 721 CAGATGAACC ACCCTCCTCT TCCCGATCCC TTGGGCCGGC CCGATGAAGA TAGTTCCTCT
 781 TCGTCTTCCT CCTGCAGTTC GGCTTCGGAC TCGGAGAGTG AGTCCGAGGA GATGAAATGC
 841 AGCAGTGGCG GAGGAGCATC CGTGACCTCG AGCCACCATG GGCGCGGCGG TTTTGGTGGC
 901 GCGGCCTCCT CCTCTCTGCT GAGCTGCGGC CATCAGAGCA GCGGCGGGGC GAGCACCGGA
 961 CCCCGCAAGA AGAAGAGCAA ACGCATCTCC GAGTTGGACA ACGAGAAGGT GCGCAATATC
1021 ATGAAAGATA AGAACACCCC CTTCTGCACA CCCAACGTGC AGACTCGGCG GGGTCGCGTC
1081 AAGATTGACG AGGTGAGCCG CATGTTCCGC AACACCAATC GCTCTCTTGA GTACAAGAAC
1141 CTGCCCTTCA CGATTCCCAG TATGCACCAG GTGTTAGATG AGGCCATCAA AGCCTGCAAA
1201 ACCATGCAGG TGAACAACAA GGGCATCCAG ATTATCTACA CCCGCAATCA TGAGGTGAAG
1261 AGTGAGGTGG ATGCGGTGCG GTGTCGCCTG GGCACCATGT GCAACCTGGC CCTCTCCACT
1321 CCCTTCCTCA TGGAGCACAC CATGCCCGTG ACACATCCAC CCAAAGTGGC GCAGCGCACA
1381 GCCGATGCTT GTAACGAAGG CGTCAAGGCC GCGTGGAGCC TCAAAGAATT GCACACCCAC
1441 CAATTATGCC CCCGTTCCTC CGATTACCGC AACATGATCA TCCACGCTGC CACCCCCGTG
1501 GACCTGTTGG GCGCTCTCAA CCTGTGCCTG CCCCTGATGC AAAAGTTTCC CAAACAGGTC
1561 ATGGTGCGCA TCTTCTCCAC CAACCAGGGT GGGTTCATGC TGCCTATCTA CGAGACGGCC
1621 ACGAAGGCCT ACGCCGTGGG GCAGTTTGAG CAGCCCACCG AGACCCCTCC CGAAGACCTG
1681 GACACCCTGA GCCTGGCCAT CGAGGCAGCC ATCCAGGACC TGAGGAACAA GTCTCAGTAA
```

Fig. 14

Western Blot

Fig. 15A

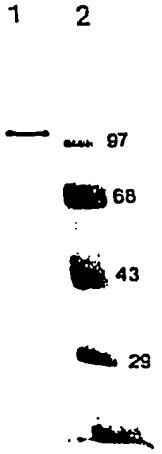
Lane 1 SF9 insect cell lysate
Lane 2 Baculovirus RCMVIE1 infected SF9 cell lysate
Lane 3 RCMVIE1 purified protein preparation
Lane 4 Baculovirus RCMVIE2 infected SF9 cell lysate
Lane 5 RK-13 cells
Lane 6 vP1479 infected RK-13 cell lysate
Lane 7 Prestained Molecular Weight Markers



15B

Coomassie Blue Stained Gel

Lane 1 RCMVIE1 purified protein preparation
Lane 2 Prestained Molecular Weight Markers



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02191

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : A61K 38/00, 39/245, 39/25, 39/295
US CL : 424/230.1, 231.1, 202.1, 229.1; 514/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/230.1, 231.1, 202.1, 229.1; 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,534,258 A (GOLUBEV et al) 09 July 1996, see entire document.	1-10
Y	MELNICK, J. L. et al. Cytomegalovirus and atherosclerosis. Bioessays. 1995, Vol. 17, No. 17, pages 899-903, see entire document.	1-9
A	VERCELLOTTI, G. M. Potential Role of Viruses in Thrombosis and Atherosclerosis. Trends in Cardiovascular Medicine. July/August 1995, Vol. 5, No. 4, pages 128-133, see entire document.	1-9
A	SPIER, E. et al. Potential Role of Human Cytomegalovirus and p53 interaction in Coronary Restenosis. Science. 15 July 1994, Vol. 265, pages 391-394, see entire document.	1-70

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"P"	document published prior to the international filing date but later than the priority date claimed		

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